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Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor

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Although many proteins have been shown to participate in ligand-stimulated endocytosis of EGF receptor (EGFR), the adaptor protein responsible for interaction of activated EGFR with endocytic machinery remains elusive. We show here that EGF stimulates transient tyrosine phosphorylation of Tom1L1 by the Src family kinases, resulting in transient interaction of Tom1L1 with the activated EGFR bridged by Grb2 and Shc. Cytosolic Tom1L1 is recruited onto the plasma membrane and subsequently redistributes into the early endosome. Mutant forms of Tom1L1 defective in Tyr-phosphorylation or interaction with Grb2 are incapable of interaction with EGFR. These mutants behave as dominant-negative mutants to inhibit endocytosis of EGFR. RNAi-mediated knockdown of Tom1L1 inhibits endocytosis of EGFR. The C-terminal tail of Tom1L1 contains a novel clathrin-interacting motif responsible for interaction with the C-terminal region of clathrin heavy chain, which is important for exogenous Tom1L1 to rescue endocytosis of EGFR in Tom1L1 knocked-down cells. These results suggest that EGF triggers a transient Grb2/Shc-mediated association of EGFR with Tyr-phosphorylated Tom1L1 to engage the endocytic machinery for endocytosis of the ligand–receptor complex.

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Introduction

Endocytosis of surface proteins occurs either constitutively (e.g. the transferrin receptor, TfR) or only on ligand binding (e.g. the EGF receptor, EGFR). Clathrin mediates endocytosis of many surface proteins (Kirchhausen, 2000; Le Roy and Wrana, 2005; Roth, 2006), which is generally facilitated by adaptor proteins that bind simultaneously to cargo proteins and clathrin. Many accessory proteins also participate in endocytosis (Slepnev and De Camilli, 2000; Traub, 2003). AP-2, β-arrestin, autosomal recessive hypercholesterolemia (ARH) and disabled-2 (Dab2) are examples of known adaptors mediating endocytosis of various surface proteins. AP-2, a heterotetrameric protein complex consisting of small σ2, medium μ2, and large α and β2 subunits, is involved in endocytosis of many surface proteins such as TfR through binding to double leucine or Tyr-based endocytic motifs in the cytoplasmic domain of the surface proteins (Bonifacino and Lippincott-Schwartz, 2003; Traub, 2003; Robinson, 2004). β-arrestins are specialized adaptors responsible for endocytosis of G-protein coupled receptors such as β2-adrenergic receptor (Lefkowitz and Shenoy, 2005; Moore *et al*, 2007; Wolfe and Trejo, 2007). ARH and Dab2 are specific adaptors responsible for endocytosis of the LDL receptor (Keyel *et al*, 2006; Maurer and Cooper, 2006).

EGFR is an example of a family of surface proteins termed as receptor tyrosine kinases (RTKs) (Blume-Jensen and Hunter, 2001; Schlessinger, 2004; Citri and Yarden, 2006). Ligand binding generally causes dimerization of the receptor and consequent activation of the tyrosine kinase activity of the intracellular cytoplasmic domain to mediate intracellular signalling. Ligand binding also induces endocytosis of the activated receptor. Overexpression of EGFR or its defective endocytosis because of mutations is causally linked to several human cancers (Peschard and Park, 2003; Citri and Yarden, 2006; Lo *et al*, 2006; Sharma *et al*, 2007). Endocytosis of EGFR is mediated by clathrin, but several studies have suggested that AP-2 does not have a major function in EGF-stimulated clathrin-dependent endocytosis of EGFR (Nesterov *et al*, 1999; Conner and Schmid, 2003; Motley *et al*, 2003; Traub, 2003; Lakadamyali *et al*, 2006; Sorkin and Goh, 2009), suggesting the existence of additional or alternative adaptors responsible for endocytosis of EGFR. Identification of such adaptors will greatly enhance our understanding of the emerging function of endocytosis in cellular signalling and endocytic sorting (Traub, 2003; Le Roy and Wrana, 2005; Citri and Yarden, 2006; Deinhardt *et al*, 2006; Lakadamyali *et al*, 2006; Polo and Di Fiore, 2006).

Tom1L1 belongs to the VHS domain-containing family of proteins, which also include Hrs, Stam1, Stam2, GGA1, GGA2, GGA3, Tom1 and Tom1L2 (Lohi *et al*, 2002; Bonifacino, 2004). Characterized by an N-terminal VHS domain followed by a central GAT domain and a C-terminal region, Tom1, Tom1L1 and Tom1L2 together form a subfamily of VHS domain proteins. Tom1 is known to be recruited to the endosome by endofin (an endosomal FYVE domain protein) and can subsequently recruit clathrin onto the endosome (Seet *et al*, 2004; Seet and Hong, 2005). Tom1L1 is Tyr-phosphorylated by the Src family kinases and may have

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a negative function in Src kinase signalling (Seykora *et al*, 2002; Franco *et al*, 2006). Furthermore, Tom1L1 can interact with Hrs and TSG101/VPS23, which are important for sorting of endocytosed EGFR into multivesicular bodies (MVBs) for delivery to the lysosome for degradation (Puertollano, 2005). In this report, we provide evidence showing that Tom1L1 is a regulated adaptor bridging activated EGFR with the endocytic machinery for internalization of activated EGFR.

Results

Src family kinases, stimulated by EGF, catalyse Y460-dependent tyrosine phosphorylation of Tom1L1 to mediate interaction with Grb2

Mouse Tom1L1 is phosphorylated by the Src kinase family (Seykora *et al*, 2002). Human Tom1L1, but not its related Tom1 or Tom1L2, is also tyrosine phosphorylated by Src (Supplementary Figure S1A and B). Several mutants of Tom1L1 were created to define the residue responsible for its Tyr-phosphorylation, including Tom1L1–SH3 in which the potential SH3 domain-binding motif (^{421}LPPLP) was mutated into AAAAA, Tom1L1/Y392F in which Y392 was mutated to F to disrupt the potential Grb2-binding motif, Tom1L1/Y460F in which Y460 was mutated to F to disrupt the potential Src-phosphorylation motif and Tom1L1/Y460F–SH3 in which both the SH3-interacting motif and Src target motif were mutated (Figure 1A). Examining these mutants revealed that Y460 is necessary for phosphorylation (Supplementary Figure S1C). The Src-related Fyn kinase also phosphorylated Tom1L1, but not Tom1 or Tom1L2 (Supplementary Figure 1D). In view of the interaction of Tom1L1 with Grb2 (Seykora *et al*, 2002) and the potential function of Grb2 in endocytosis of EGFR (Johannessen *et al*, 2006), we found that Tyr-phosphorylation of Tom1L1 is important for interaction with Grb2, as Tom1L1 failed to interact with Grb2 in the absence of the kinase (Supplementary Figure S1E). Consistently, Y460F also disrupted the interaction with Grb2 (Supplementary Figure S1F). Although EGF-stimulated Tyr-phosphorylation was not affected by Y392F mutation (Supplementary Figure S1G), Y392F mutation also abolished the interaction with Grb2 (Supplementary Figure 1F). These results suggest that Src-catalysed Tyr-phosphorylation at Y460 of Tom1L1 triggers interaction with Grb2 that is also dependent on Y392. These two residues may thus act in a concerted manner for Tom1L1 to interact with Grb2. Tom1L1 is also Tyr-phosphorylated under physiological stimulations. A431 cells stably expressing HA–Tom1, Tom1L1 or Tom1L2 were treated with EGF for 5 min followed by immunoprecipitation and immunoblot. HA-tagged proteins were efficiently precipitated regardless of EGF stimulation (Figure 1B, lower panel). Probing with phospho-Tyr, 4G10 Mab showed that Tom1L1 was Tyr-phosphorylated, and this was observed only after EGF stimulation (Figure 1B, upper panel). Mutation of Y460 abolished EGF-stimulated Tyr-phosphorylation of Tom1L1 (Figure 1C). To examine whether Tom1L1 is Tyr-phosphorylated on stimulation by other ligands of RTKs, we treated NIH-3T3 cells expressing HA–Tom1L1 and its mutants with PDGF-BB (Figure 1D) or FGF2 (Figure 1E). Both PDGF-BB and FGF2 stimulated Y460-dependent Tyr-phosphorylation of Tom1L1. As Y460 is Tyr-phosphorylated by Src family kinases, we tested the possibility that Tyr-phosphorylation of Tom1L1, stimulated by EGF, PDGF-BB and FGF2, may be mediated by Src family kinases. In support of this possibility, EGF-stimulated Tyr-phosphorylation of Tom1L1 was essentially abolished in the presence of Src kinases inhibitor PP1 (Figure 1F). Furthermore, in SYF mouse cells lacking Src, Yes and Fyn, EGF, PDGF-BB or FGF2 failed to stimulate Tyr-phosphorylation of Tom1L1, in contrast to their ability to stimulate Tyr-phosphorylation of Tom1L1 in NIH-3T3 mouse cells (Figure 1G). These results suggest that EGF, PDGF and FGF stimulate Tyr-phosphorylation of Tom1L1 through Src family kinases, which are known to be activated by these ligands (Martin, 2001; Ishizawar and Parsons, 2004).

EGF-stimulated Tyr-phosphorylation of Tom1L1 is transient and correlates with its transient interaction with EGFR

We next examined the kinetics of EGF-stimulated Tyr-phosphorylation of Tom1L1. A431 cells expressing HA–Tom1L1 were stimulated with EGF for various periods of time. Cell lysates were precipitated with anti-HA antibody followed by immunoblot (Figure 2A, upper two panels). When probed with 4G10 Mab, Tom1L1 was observed to be Tyr-phosphorylated after stimulation for 2 min, with the maximal level of Tyr-phosphorylation being detected at 5 min. At 10 min, Tyr-phsophorylated Tom1L1 was significantly reduced. At 20 min and beyond, Tom1L1 was no longer phosphorylated. In addition to Tyr-phosphorylated Tom1L1, another Tyr-phosphorylated protein of about 180–200 kDa was detected by 4G10 Mab (Figure 2A, upper band of upper panel). As EGFR is about 180–200 kDa and is Tyr-phosphorylated on EGF stimulation (Supplementary Figure S2) (Zhang *et al*, 2005; Olsen *et al*, 2006), we probed the same blot with antibodies against EGFR and a similar pattern was observed (Figure 2A, second panel). These results suggest that EGF not only stimulated Tyr-phosphorylation of Tom1L1, but also triggered a transient association of Tyr-phosphorylated Tom1L1 with Tyr-phosphorylated EGFR. As co-immunoprecipitation of EGFR was not detected for Tom1L1/Y460F or Tom1L1/Y392F (Figure 2B), Tyr-phosphorylation mediated by Src family kinases and its ability to interact with Grb2 are both important for Tom1L1 to interact with activated EGFR. To study endogenous Tom1L1, we raised two specific antibodies against Tom1L1. The first antibody (anti-Tom1L1) was raised against recombinant C-terminal tail (residues 286–476) of Tom1L1 and it recognized both endogenous Tom1L1 and HA-tagged Tom1L1, but did not react with Tom1 or Tom1L2 (Supplementary Figure S3A and B). Another antibody (anti-pTom1L1) was raised using a short peptide (^{456}TEAIpYEEIDAHQHKG^{470}) in which Y460 is phosphorylated. Although the anti-pTom1L1 antibody recognized both Tyr-phosphorylated Tom1L1 and Tom1L1 (Supplementary Figure S3C), it has a higher affinity for Tyr-phosphorylated Tom1L1. When the antibody was tested in starved cells (in which no Tyr-phosphoryated Tom1L1 was present) or cells stimulated with EGF, the anti-pTom1L1 signal was decreased or increased, respectively (Supplementary Figure S3D, second panel), indicating that this antibody has a higher affinity for Tyr-phosphorylated Tom1L1 than unphosphorylated Tom1L1 (Supplementary Figure S3D, first panel, lane 2). We have used these antibodies to study the behaviour of endogenous Tom1L1. On stimulation with EGF, endogenous Tom1L1 (Figure 2C, lower band of upper panel) was Tyr-phosphorylated with similar kinetics as HA-tagged Tom1L1.

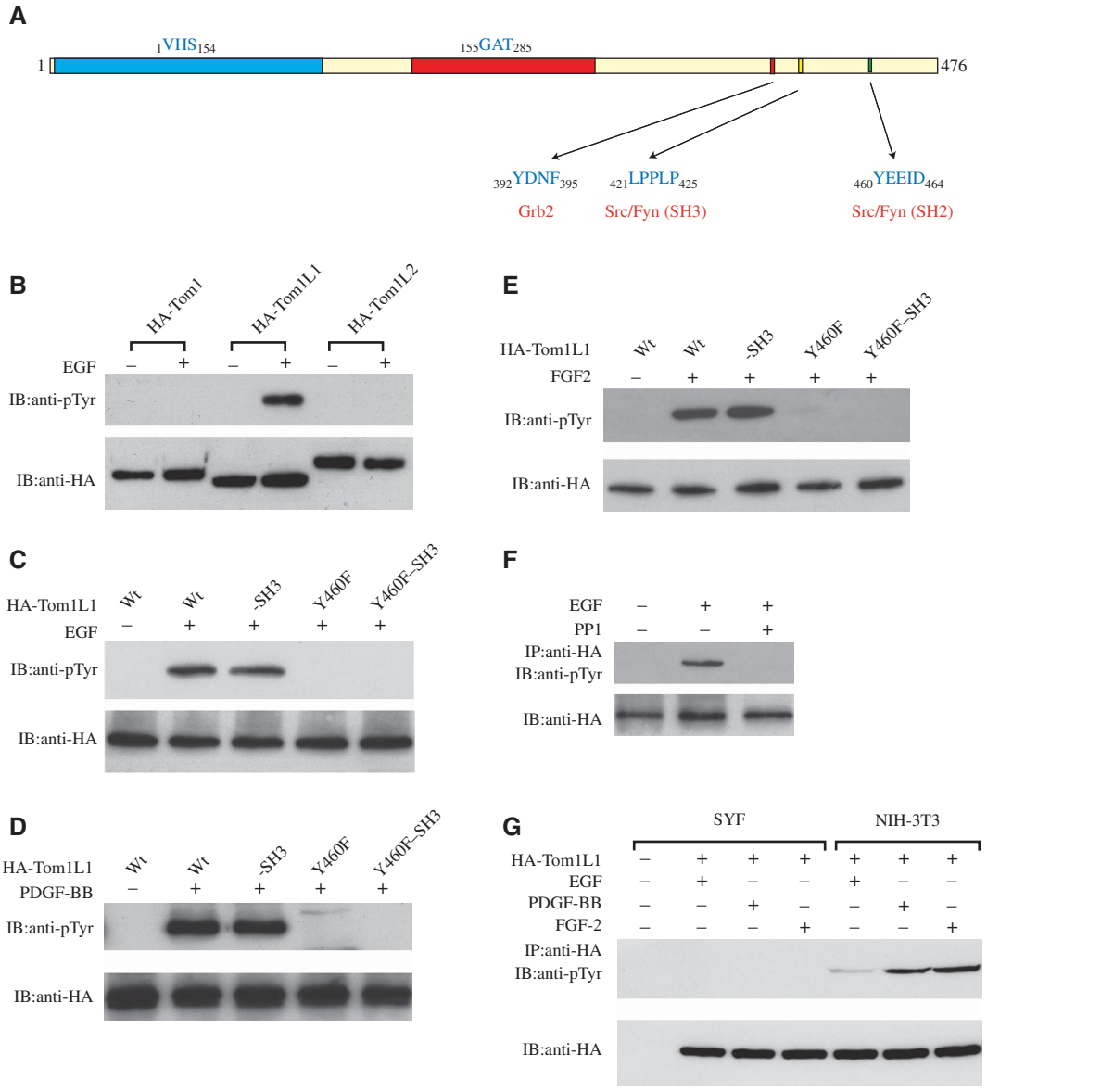


Figure 1 EGF, PDGF and FGF stimulate Src-catalysed Tyr-phosphorylation of Tom1L1. (**A**) Structural features of Tom1L1. The domains and motifs are indicated. (**B**) Starved A431 cells expressing the indicated proteins were untreated or treated with EGF (100 ng/ml for 5 min) as indicated. Anti-HA precipitates were analysed by immunoblot using the indicated antibody. (**C**–**E**) Cells expressing HA–Tom1L1 (wt) or its various mutants were either untreated or treated with EGF (100 ng/ml for 5 min) (C), PDGF-BB (50 ng/ml for 5 min) (D) or FGF2 (20 ng/ml for 5 min) (E). Anti-HA precipitates were analysed by immunoblot using the indicated antibody. (**F**) Cells expressing HA–Tom1L1 were either untreated or treated with EGF in the presence or absence of Src kinase inhibitor PP1 (10 nM). Anti-HA precipitates were analysed by immunoblot as indicated. (**G**) Mouse NIH-3T3 cells or SYF cells (defective in Src family kinases Src, Yes and Fyn) were either untransfected or transfected to express HA–Tom1L1. Cells were either untreated or treated with EGF, PDGF-BB or FGF2 as indicated. Anti-HA precipitates were analysed by immunoblot as indicated.

Furthermore, Tyr-phosphorylated EGFR (Figure 2C, upper band of upper panel and middle panel) was also transiently co-immunoprecipitated with Tom1L1 by anti-Tom1L1 antibody with kinetics correlating with the pattern of Tom1L1 Tyr-phosphorylation. The signals detected with anti-pTom1L1 antibody were also transiently increased during EGF stimulation (Figure 2C, third panel), confirming that it has a higher affinity for Tyr-phosphorylated Tom1L1. EGF stimulation thus induces transient Tyr-phosphorylation of endogenous Tom1L1 and interaction with Tyr-phosphorylated EGFR. An earlier study has shown that Grb2 can interact with EGFR indirectly through Shc (Rozakis-Adcock *et al*, 1992; Vieira *et al*, 1996), enabling the SH2 domain of Grb2 to interact with other proteins. Shc may thus mediate interaction of Tom1L1–Grb2 complex with EGFR. The importance of Grb2 and Shc in mediating the interaction of endogenous Tom1L1 with EGFR was supported by the observation that EGF-induced co-immunoprecipitation of EGFR with Tom1L1 was significantly compromised in cells transfected with siRNA to knock down the expression of Grb2 (Figure 2D, lane 4) or Shc (Figure 2D, lane 5). Simultaneous knockdown of both Grb2 and Shc led to robust inhibition of Tom1L1–EGFR interaction (Figure 2D, lane 6), as compared with cells transfected with non-targeting siRNA (Figure 2D, lane 3). Furthermore, when 13A9 Mab,

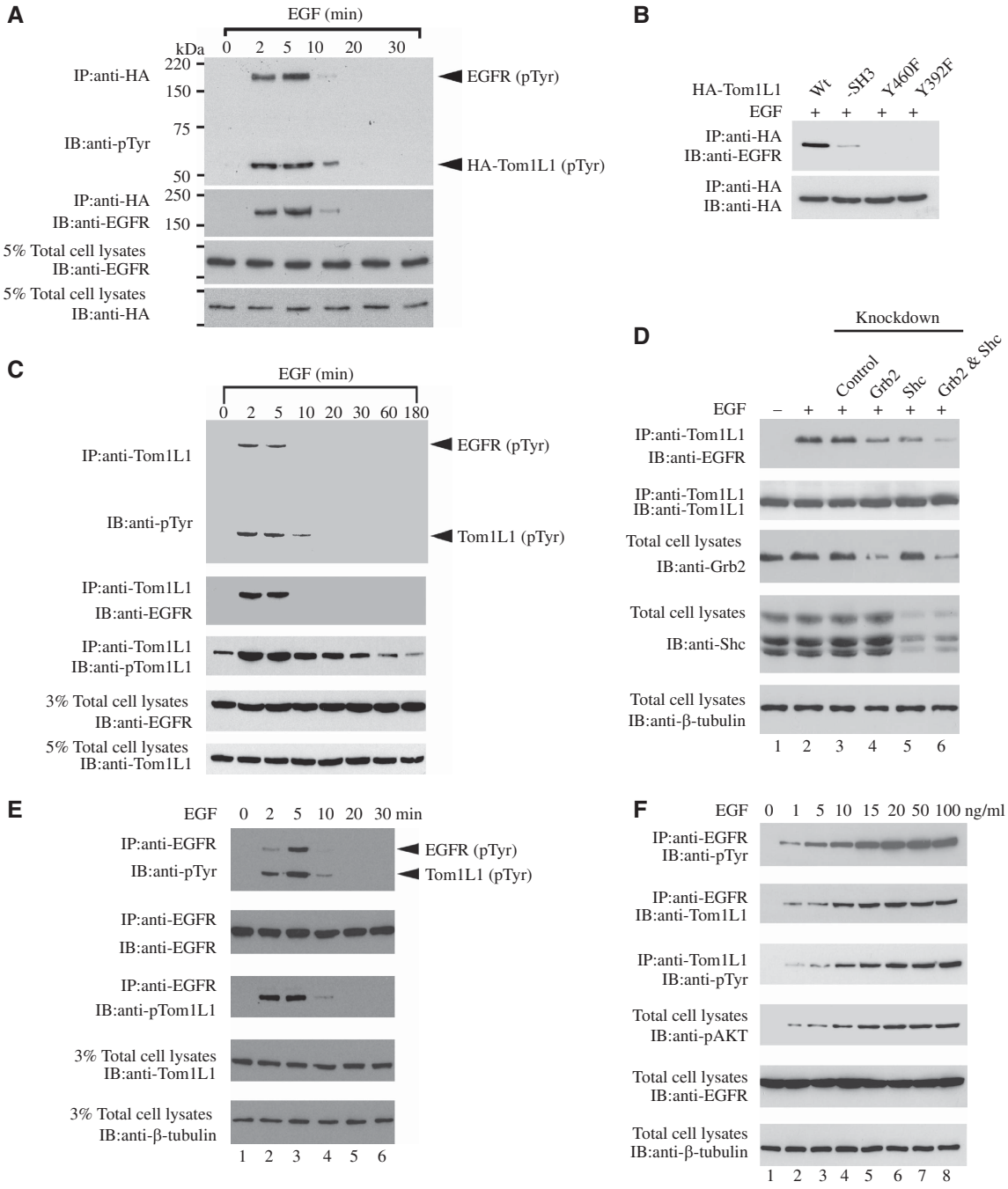


Figure 2 EGF stimulates transient association of Tyr-phosphorylated Tom1L1 with activated EGFR through Grb2/Shc. (**A**) Cells expressing HA–Tom1L1 were treated with 100 ng/ml of EGF for the indicated period of time. The cell lysates (lower two panels) and anti-HA precipitates (upper two panels) were analysed by immunoblot using the indicated antibody. (**B**) Cells expressing indicated proteins were treated with EGF (100 ng/ml for 5 min). Anti-HA precipitates were analysed by immunoblot as indicated. (**C**) A431 cells were treated with EGF (100 ng/ml) for the indicated period of time. Anti-Tom1L1 precipitates were analysed by immunoblot using the indicated antibody. Cell lysates were also analysed (bottom two panels). (**D**) Control A431 cells (lanes 1–2) or cells transfected with control non-targeting siRNA (lane 3) or siRNA-targeting Grb2 (lane 4), Shc (lane 5) or both (lane 6) were either untreated (lane 1) or treated with EGF (100 ng/ml for 5 min) (lanes, 2–6). Anti-Tom1L1 precipitates were analysed to detect the co-immunoprecipitated EGFR (the upper panel) and Tom1L1 (the second panel). The cell lysates were also analysed using the indicated antibody (bottom three panels). (**E**) Starved A431 cells were incubated with the 13A9 antibody (20 μg/ml) for 1 h at 0°C. After washing, the cells were either untreated (lane 1) or treated (lanes 2–6) with EGF (100 ng/ml) for the indicated period of time. The 13A9–EGFR complex was recovered from cell lysates and analysed by immunoblot using the indicated antibody (first three panels). Cell lysates (bottom two panels) were also analysed. (**F**) A431 cells expressing HA–Tom1L1 were treated with indicated dose of EGF (0–100 ng/ml) for 5 min. Anti-EGFR precipitates were analysed by immunoblot using anti-phospho-Tyr antibody (upper panel) and anti-Tom1L1 antibody (second panel). Anti-Tom1L1 precipitates were also analysed using anti-phospho-Tyr antibody (third panel). The cell lysates were also analysed using the indicated antibody.

which recognizes extracellular domain of EGFR and does not interfere with EGF binding to EGFR and signalling (Sigismund *et al*, 2005), was first incubated with intact A431 cells followed by EGF stimulation for 0–30 min, endogenous Tom1L1 was co-recovered with 13A9–EGFR complex (Figure 2E, lanes 2–4) during the 2–10 min interval after EGF

stimulation. This result suggests that surface EGFR in intact cells interacts with endogenous Tom1L1 during 2–10 min of EGF stimulation. To gain insight into the dose-dependent effect, A431 cells stably expressing HA–Tom1L1 were treated with increasing dose of EGF (0–100 ng/ml) for 5 min. Tom1L1 (Figure 2F, second panel) was recovered with Tyr-phosphorylated EGFR by co-immunoprecipitation with anti-EGFR antibody, which is correlated with the pattern of EGFR Tyr-phosphorylation. Similar result was observed by immunoprecipitation using anti-Tom1L1 antibody followed by probing with 4G10 Mab (Figure 2F, third panel). The activation of Akt1 exhibited a similar dose-dependent pattern (Figure 2F, panel 4). These results suggest that 15–20 ng/ml of EGF can achieve optimal response that is comparable to that elicited by 100 ng/ml of EGF with regards to Tyr-phosphorylation of EGFR, Tyr-phosphorylation of Tom1L1, interaction of Tom1L1 with EGFR and activation of Akt1 in A431 cells. At lower doses (1–10 ng/ml) of EGF, EGFR is only partially tyrosine phosphorylated and not fully competent for stimulating Tyr-phosphorylation of Tom1L1, inducing interaction with Tom1L1, or downstream signalling leading to Akt1 activation (Figure 2F).

EGF induces recruitment of Tom1L1 from the cytoplasm to the plasma membrane and subsequent redistribution with EGFR into the early endosome

Endogenous Tom1L1 is primarily present in the cytoplasm (Supplementary Figure S4A–C). We then examined the effect of EGF stimulation on the distribution of endogenous Tom1L1. Starved A431 cells were treated with EGF for various periods of time and then processed for immunofluorescence microscopy using anti-pTom1L1 antibody. Tom1L1 was initially detected in the cytoplasm, but was observed to be enriched on the plasma membrane after a 2–5 min treatment with EGF (Figure 3A) and co-localized noticeably with EGFR and clathrin in A431 cells (Figure 3B). This result was also confirmed in Hela cells (Supplementary Figure S4D). The shift in localization corresponded well with the time of induction of Tyr-phosphorylated Tom1L1 and its interaction with EGFR. After 10 min of EGF treatment, Tom1L1 was located at both the plasma membrane and in punctate structures beneath the membrane (Figure 3A). After 20–30 min, Tom1L1 became enriched in punctate structures characteristic of endosomes. Tom1L1 exhibited cytosolic distribution after prolonged treatment (180 min) with EGF. The structures marked by Tom1L1 during the 20–30 min period were also enriched for the early endosomal marker, EEA1, but segregated away from the structures marked by the late endosomal and lysosomal marker, LAMP1 (Figure 3C). These results suggest that EGF stimulated the recruitment of cytosolic Tom1L1 onto the plasma membrane whereupon Tom1L1 was Tyr-phosphorylated and complexed with EGFR. The membrane pool of Tom1L1 then redistributed with EGFR into the early endosome. As Tom1L1 was no longer phosphorylated after 20 min of stimulation, the retention of the membrane pool of Tom1L1 on the early endosome during the process of EGFR endocytosis is probably not dependent on its Tyr-phosphorylation. We speculate that this is likely mediated by its interaction with other endosomal proteins such as Hrs and/or TSG101, which are known to interact with Tom1L1 (Puertollano, 2005).

Mutant forms of Tom1L1 defective in Tyr-phosphorylation and/or interaction with Grb2 inhibit endocytosis of EGFR

The functional significance of EGF-stimulated Tyr-phosphorylation of Tom1L1 and its interaction with EGFR was investigated. First, the effect of various mutants of Tom1L1 was examined. Cells were incubated with Texas Red-EGF on ice for 60 min to allow binding to surface EGFR. After washing, cells were incubated at 37°C for 3 h to allow its internalization and delivery to the lysosome for degradation. To visualize surface-bound Texas Red-EGF, cells were fixed immediately after washing. Under this condition, A431 cells and A431 cells expressing HA–Tom1L1, Tom1L1–SH3, Tom1L1/Y460F all exhibited comparable levels of binding of Texas Red-EGF (Figure 4A, left panels), which has a characteristic surface appearance highlighted by the labelling of cell boundaries. After incubation at 37°C for 3 h, the majority of Texas Red-EGF was essentially degraded in A431 cells as well as in A431 cells expressing HA–Tom1L1 and Tom1L1–SH3 with trace amounts on the plasma membrane and in intracellular punctates (Figure 4A, right panels). However, in cells expressing Tom1L1/Y460F, a significant amount of Texas Red-EGF remained detectable on the plasma membrane and intracellular punctate structures. Similar blockage of endocytosis and degradation of Texas Red-EGF was seen in cells expressing Tom1L1/Y392F and Tom1L1/Y460F–SH3 (data not shown). We next examined EGFR degradation induced by EGF. Cells were incubated with EGF for 0 min or 3 h at 37°C and total cell lysates were used for immunoblot (Figure 4B). Densitometric analysis of the immunoblots was performed (Figure 4C). A comparison on the amount of EGFR in the control A431 cells at 0 versus 3 h (Figure 4B, lane 1, first and second panel) indicated that about 80% of EGFR was degraded after 3 h at 37°C, as only about 20% of EGFR was detected. In cells stably expressing HA-tagged Tom1L1 (lane 2) and Tom1L1–SH3 (lane 3), EGFR degradation was slightly but reproducibly enhanced because around 15% EGFR was detected. In cells expressing HA-tagged Tom1L1/Y460F (lane 4), Tom1L1/Y460F–SH3 (lane 5) and Tom1L1/Y392F (lane 6), EGFR degradation was significantly impaired as about 50% of EGFR remained. These results suggest that Tom1L1/Y460F, Tom1L1/Y460F–SH3 and Tom1L1/Y392F, which are no longer able to associate with activated EGFR because of defects in Tyr-phosphorylation and/or interaction with Grb2, significantly blocked internalization/degradation of Texas Red-EGF and inhibited EGF-stimulated degradation of EGFR (Figure 4B and C).

To directly define a function for Tom1L1 in the internalization of EGFR, we monitored by immunofluorescence microscopy, the distribution of activated EGFR using an antibody (anti-pEGFR), which preferentially recognizes Tyr-phosphorylated EGFR (this antibody also recognizes unphosphorylated EGFR with less efficiency, Supplementary Figure S2, second panel). EGFR was initially detected on the cell surface in both A431 cells and cells expressing various forms of Tom1L1 (Figure 5A, left panels). After stimulation with EGF for 20 min, EGFR was mainly distributed in intracellular vesicular structures with some being detected on the surface in A431 cells (Figure 5A, panel d). Similarly, EGFR was predominantly detected in the intracellular vesicular structures in cells expressing Tom1L1 (panel h) or Tom1L1–SH3 (panel l) with noticeably less amounts

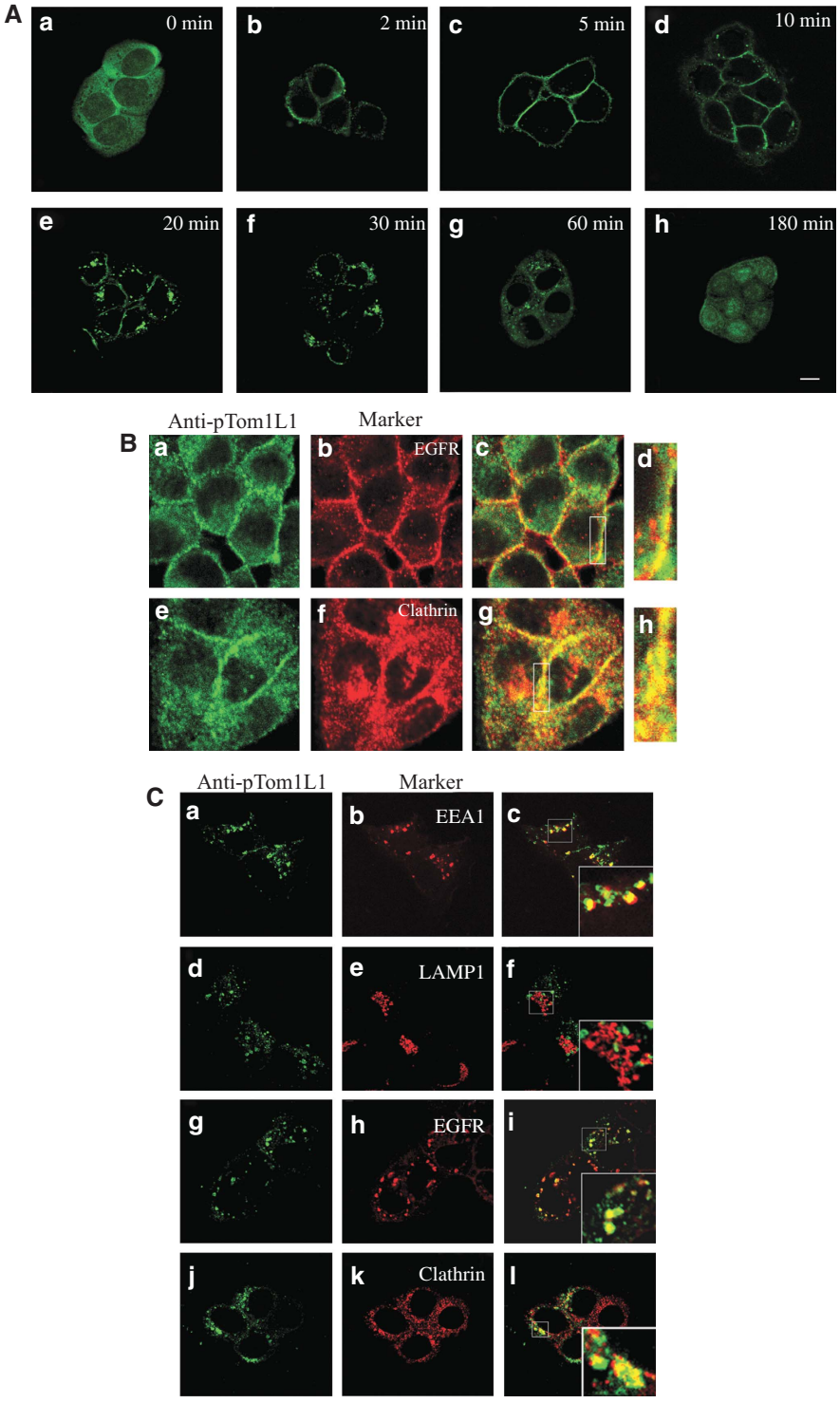


Figure 3 EGF stimulates recruitment of Tom1L1 from the cytoplasm to the plasma membrane followed by redistribution to the endosome. (**A**) A431 cells treated with EGF (100 ng/ml) for the indicated period of time were analysed by immunofluorescence microscopy using anti-pTom1L1 antibody. Bar, 10 μM. (**B**) A431 cells treated with EGF (100 ng/ml) for 2 min were analysed by indirect immunofluorescence microscopy using anti-pTom1L1 antibody and antibodies against the indicated proteins. Bar, 10 μM. (**C**) A431 cells treated with EGF (100 ng/ml) for 30 min were analysed by immunofluorescence microscopy using anti-pTom1L1 and antibodies against the indicated proteins. Bar, 10 μM.

being detected on the surface. In contrast, EGFR was prominently detected on the cell surface in cells expressing Tom1L1/Y460F (panel p) or cells expressing Tom1L1/Y392F or Tom1L1/Y460F–SH3 cells (data not shown). These results suggest that Tom1L1/Y460F, Tom1L1/Y392F and Tom1L1/Y460F–SH3 inhibited EGFR endocytosis. We next monitored the internalization of surface-biotinylated EGFR in response to EGF (Figure 5B) followed by quantitative analysis by densitometry (Figure 5C), as biotinylation is widely used to selectively mark surface-exposed proteins including EGFR

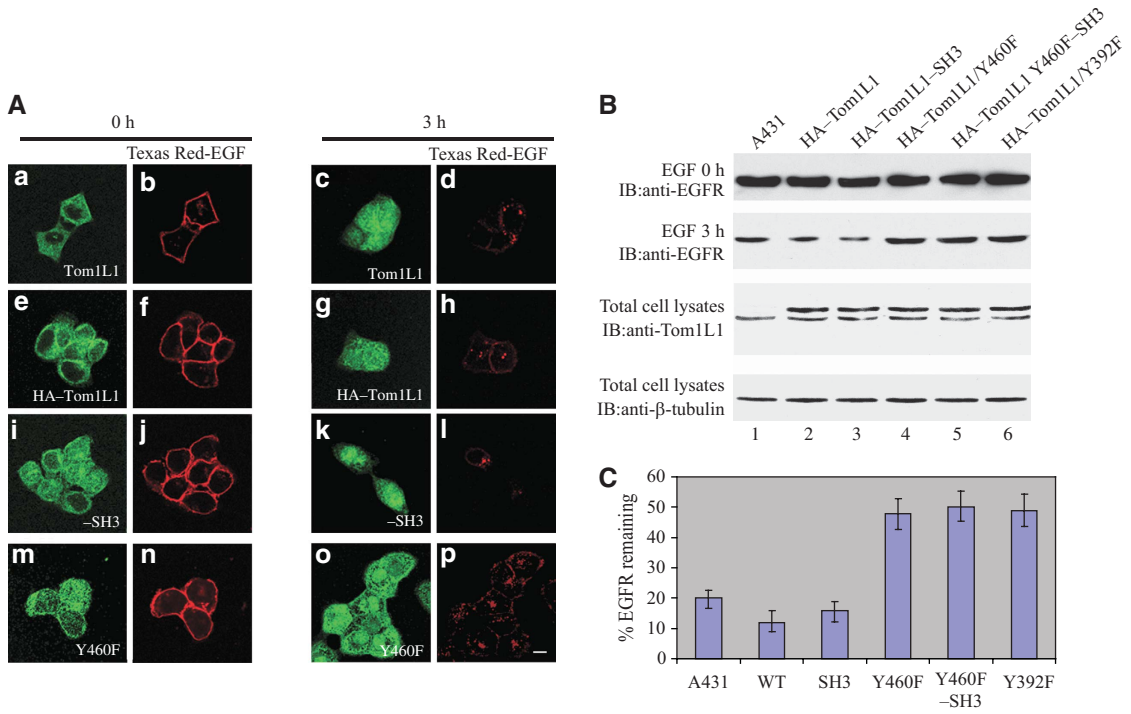


Figure 4 Tom1L1 mutants defective in Tyr-phosphorylation and/or interaction with Grb2 delay degradation of EGF and EGFR. (**A**) A431 cells or A431 cells expressing HA–Tom1L1 or its mutants as indicated were incubated with Texas Red-EGF (20 ng/ml) on ice for 60 min. After washing, the cells were incubated at 37°C for 0 h (left panels) or 3 h (right panels). Cells were analysed by immunofluorescence microscopy to detect Tom1L1 and to visualize Texas Red-EGF. Bar, 10 μM. (**B**) Cells as indicated were incubated with EGF (100 ng/ml) at 37°C for 0 or 3 h. Cell lysates were analysed by immunoblot to detect EGFR at 0 h (upper panel) or 3 h (the second panel), Tom1L1 at 3 h (the third panel, in which both endogenous Tom1L1, the lower band, and HA-Tom1L1, the upper band, were detected), and β-tubulin at 3 h (bottom panel). (**C**) The results from three independent experiments exampled in (B) were quantified by densitometric analysis for EGFR levels at 0 and 3 h. After being normalized against the level of β-tubulin, the averages of the percentage of EGFR detected at 3 h (relative to the EGFR level at 0 h being set as 100%) are presented as mean ± s.d.

without reported alterations of their trafficking or function (Choi *et al*, 2004; Elia, 2008). Cells were surface biotinylated with a cleavable biotin to biotinylate surface proteins. After washing, biotinylated EGFR was stimulated with EGF to induce internalization at 37°C for various periods of time. Cells were then quickly chilled on ice to stop additional endocytosis and then treated with membrane-impermeable reducing agents to remove biotin attached to surface-exposed EGFR in a process referred to as surface stripping. The biotin attached to EGFR molecules that have already been internalized is resistant to surface stripping. Biotinylated EGFR can be retrieved by straptavidine beads for detection by immunoblot. In this assay, the amount of biotinylated EGFR that is resistant to surface stripping serves as a quantitative and direct measure of the amount of internalized EGFR. Using this approach, essentially all surface-biotinylated EGFR was sensitive to the surface stripping at the beginning of EGF stimulation (lane 2). Within 2 min of stimulation, about 60% of EGFR had become resistant to surface stripping (lane 3), suggesting that the majority of EGFR had been sequestered into clathrin-coated vesicles that were no longer accessible to externally applied reducing agents. The amount of internalized EGFR increased gradually over 5–20 min (lanes 4–5) with >95% of biotinylated EGFR becoming resistant to surface stripping after 20 min (lane 6). Similar kinetics of endocytosis was observed in cells expressing Tom1L1 or Tom1L1–SH3. In cells expressing Tom1L1/Y460F, the majority (about 80%) of EGFR remained sensitive to surface stripping after 2 min of stimulation because only around 20% of EGFR became resistant to the stripping. The amount of internalized EGFR increased gradually over 5–20 min period with about 70% of EGFR being internalized after 20 min of stimulation. Similar delay of EGFR endocytosis was observed in cells expressing Tom1L1/Y392F or Tom1L1/Y460F–SH3 (Figure 5C). Hence, the most dramatic inhibition of EGFR internalization occurred at 2 min with 60% being internalized in control cells compared with 20% being internalized in cells expressing Tom1L1/Y460F, Tom1L1/Y392F or Tom1L1/Y460F–SH3. Tom1L1 may thus have a crucial function in the early events of EGFR endocytosis when activated EGFR is being sorted and sequestered into clathrin-coated pits/vesicles.

siRNA-mediated knockdown of Tom1L1 inhibits endocytosis of EGFR

To verify a function for Tom1L1 in endocytosis by an independent approach, we used siRNA-mediated knockdown to reduce the expression of endogenous Tom1L1 and to examine the consequence on EGFR degradation (Figure 6A and B) and endocytosis (Figure 6C and D). After stimulation with EGF for 3 h, about 80% of EGFR was degraded in cells transfected with either non-targeting siRNA or cells treated with siRNA-targeting GAPDH (Figure 6A, lanes 1–2). However, EGFR degradation was compromised in cells transfected with siRNA-targeting Tom1L1 with only 50% of EGFR being degraded (Figure 6A, lane 3). The endocytosis of

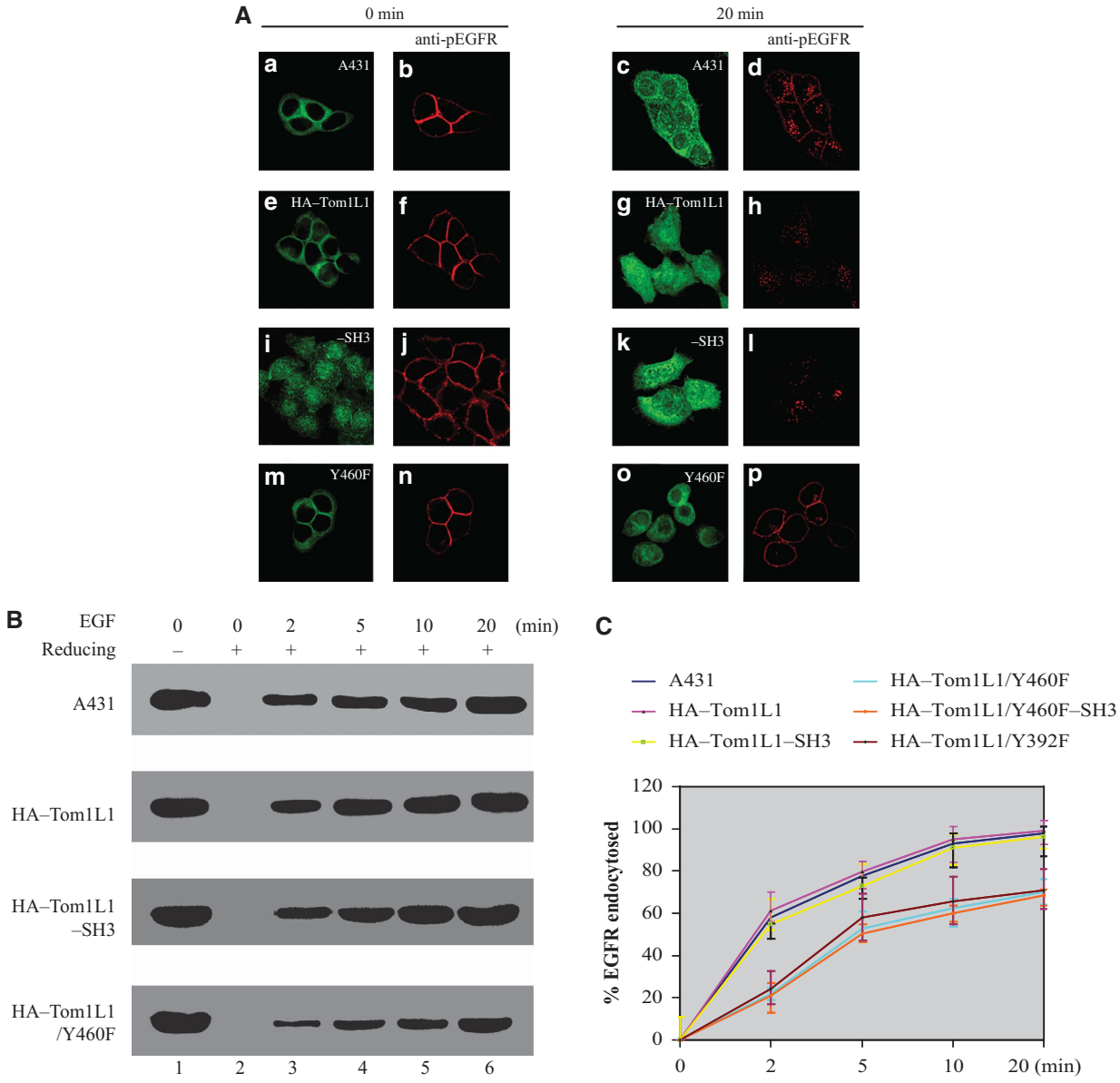


Figure 5 Tom1L1 mutants defective in Tyr-phosphorylation and/or interaction with Grb2 delay EGF-induced endocytosis of EGFR. (**A**) A431 cells expressing indicated proteins were incubated with EGF (100 ng/ml) followed by incubation at 37°C for 0 min (left panels) or 20 min (right panels) and analysed by immunofluorescence microscopy to detect Tom1L1 and EGFR. Bar, 10 μM. (**B**) A431 cells expressing indicated proteins were surface biotinylated with sulfo-NHS-SS-biotin. After incubation at 37°C with EGF (100 ng/ml) for the indicated period of time, surface-exposed biotin was removed by stripping in reducing buffer. Cell lysates were incubated with streptavidin beads to recover biotinylated proteins that have been endocytosed and inaccessible to surface stripping. The total amount of biotinylated EGFR at 0 min was also recovered. The precipitates were analysed by immunoblot to detect EGFR. The amount of biotinylated EGFR that is resistant to surface stripping is a reflection of the amount of endocytosed EGFR. (**C**) The results from three independent experiments as exampled in (B) were quantified by densitometric analysis. Each signal is normalized against the level of β-tubulin detected at each time point (not shown). Values, expressed as mean ± s.d., represent the fraction of biotinylated EGFR recovered at each time point relative to total EGFR biotinylated at 0 min.

surface-biotinylated EGFR was also monitored (Figure 6C and D). In cells transfected with the non-targeting siRNA or siRNA for GAPDH, about 60% of EGFR was internalized after EGF stimulation for 2 min (lane 3). However, the majority of EGFR was not internalized in cells transfected with Tom1L1 siRNA as only 20% of the biotinylated EGFR was protected from the surface stripping. The kinetics of EGFR internalization in cells treated with Tom1L1 siRNA (Figure 6D) was similar to that observed in cells expressing dominant-negative mutants of Tom1L1 (Figure 5C). To verify that the observed defect in EGFR endocytosis is due to the silencing of Tom1L1 by its siRNA, we examined the ability of RNAi-resistant Tom1L1 cDNA to restore endocytosis of EGFR. Significantly, the defect of EGFR endocytosis in Tom1L1 siRNA-transfected cells could be rescued by siRNA-resistant cDNA encoding mouse Tom1L1 (Figure 6E, second panel, lane 3; Figure 6F), but not by cDNA encoding human Tom1 (Figure 6E, second panel, lane 4; Figure 6F). The function of Tom1L1 in EGFR endocytosis is specific as knockdown of Tom1 or Tom1L2 did not affect EGF-stimulated endocytosis of EGFR (Figure 6G and H). These results suggest that endogenous Tom1L1 has an important and specific function in EGFR endocytosis.

C-terminal tail of Tom1L1 harbours a novel clathrin-interacting motif

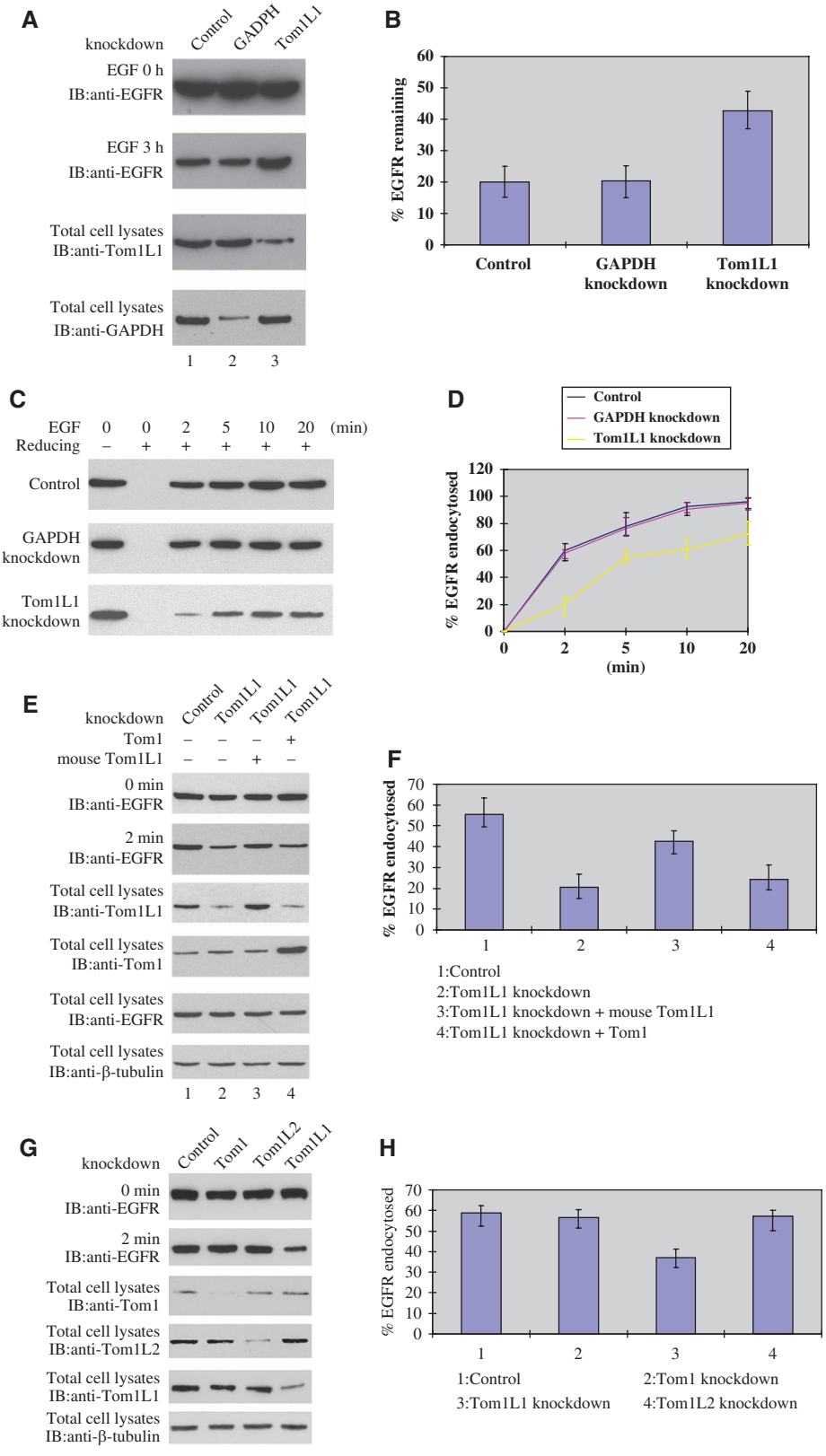
Tom1 contains a well-defined clathrin-binding motif in the C-terminal region (Yamakami *et al*, 2003; Seet and Hong, 2005) and Tom1L1 and Tom1L2 are also able to bind clathrin through their C-terminal tails (Katoh *et al*, 2006). We attempted to define a region of Tom1L1 necessary for interaction with clathrin as assessed by GST-pull down experiments using various fusion proteins of Tom1L1 (depicted schematically in Figure 7A) to retrieve clathrin heavy chain (CHC) from A431 cell cytosol. GST–Tom1L1(316–476) was able to retrieve CHC (Figure 7B, lane 3). Truncation experiments revealed that the region between residues 316 and 419 was not important for interaction with CHC (7B, lanes 4–7), suggesting that the C-terminal 57-residue tail (residues 420–476) is sufficient for interaction with CHC. This region does not contain a conventional clathrin-binding motif for N-terminal region of CHC (Dell'Angelica, 2001; Drake and Traub, 2001; Miele *et al*, 2004). Analysis of another set of C-terminally truncated deletion mutants of GST–Tom1L1 revealed that the region encompassing residues 286–450 was able to interact with CHC (Figure 7C, lane 4), whereas the fragment consisting of residues 286–441 was defective in interaction with CHC (Figure 7C, lane 3), suggesting that the residues 441–450 are necessary for CHC interaction. These observations narrowed the critical clathrin-binding residues down to a 31-residue region comprising residues 420–450 (Supplementary Figure S5A, blue box). Within this 31-residue region are the LPPLP motif predicted in interaction with SH3 domain and YEVM motif predicted in interaction with p85 subunit of PI3K. To define residues within this region important for interaction with clathrin, we have focused on other residues in this region while keeping these motifs intact. We have chosen three stretches (Supplementary Figure S5A, purple boxes) with four residues each for further examination. Using GST–Tom1L1 (286–476) as the framework, we performed site-directed mutagenesis in these three stretches by changing all four residues of each stretch into Ala and examining the consequence on interaction with clathrin. This analysis led to the identification of the ^{447}FDPL^{450} motif being important for CHC interaction (Figure 7D). The region centred at FDPL motif does not share sequence homology with consensus clathrin-binding boxes, suggesting that this is likely part of a novel clathrin-binding motif. Ala-scanning mutagenesis of each residue of the VMEFDPL stretch suggests that changing individual residue did not affect the interaction with clathrin (Supplementary Figure 5B), which is in marked contrast with canonical clathrin-binding motif such as that of Ack1 in that changing each residue of the clathrin box abolished interaction with clathrin (Teo *et al*, 2001). These results support the notion that FDPL is part of a novel clathrin-interacting module. To define the region of CHC that is involved in interaction with Tom1L1, we have produced six Myc-tagged fragments corresponding to different overlapping regions of CHC using the *in vitro* TNT transcription and translation system (Figure 7E). The translation reactions were then incubated with immobilized GST–Tom1L1(285–476) and GST–Tom1L1(285–476) FDPL450 AAAA to identify the CHC region capable of interacting with Tom1L1 in a ^{447}FDPL^{450}-dependent manner. The *in vitro* translated Myc-tagged fragments encompassing residues 1–363 (Figure 7E, lane 1), residues 327–542 (lane 4), residues 532–834 (lane 7), residues 824–1129 (lane 10), residues 1121–1335 (lane 13) and residues 1325–1675 (lane 16) were all detected by anti-Myc antibodies. When these translated reactions were incubated with immobilized GST-fusion proteins, only the C-terminal fragment (residues 1325–1675) was efficiently retained by GST–Tom1L1(285–476) (lane 18), but not by FDPL450AAAA mutant (lane 17). The results suggest that the C-terminal region of CHC is able to interact with Tom1L1. As CHC fragment consisting residues 1325–1675 purified by immunoprecipitation was also able to interact with immobilized GST–Tom1L1 (285–476) (Figure 7F), the interaction of Tom1L1 with the C-terminal region of CHC is likely to be direct. Consistent with the fact that canonical clathrin box interacts with N-terminal region of clathrin, GST–Ack1(564–582) (Teo *et al*, 2001) was able to pull down myc-clathrin(1–363), but not myc-clathrin(1325–1675) (Figure 7G). Under the same conditions, GST–Tom1L1(438–457) was able to pull down myc-clathrin(1325–1675), but not myc-clathrin(1–363). These results suggest that the 20-residue region (438–457) (green box, Supplementary Figure 5A) of Tom1L1, but not canonical clathrin box of Ack1, is sufficient to interact with the C-terminal region of clathrin.

To establish that Tom1L1 interaction with clathrin is important for EGFR endocytosis, we performed rescue experiments in A431 cells in which Tom1L1 was knocked down. RNAi-resistant mouse cDNAs encoding wild type or various mutants of mouse Tom1L1 were introduced into the siRNA-treated A431 cells. Internalization of surface-biotinylated EGFR was assessed at 2 min on stimulation with EGF. As shown in Figure 7H and quantified in Figure 7I, Tom1L1 siRNA-treated A431 cells internalized about 20–30% EGFR (second panel, lane 2) compared with 50–60% of EGFR being internalized in control cells (lane 1). When mouse Tom1L1 was introduced by retroviral infection into the knockdown cells, internalization of EGFR was rescued significantly to about 40–50% (lane 3). When the clathrin-binding ^{446}FDPL^{449} motif was mutated in mouse Tom1L1, the mutant failed to rescue the endocytosis of EGFR in the knockdown cells (lane 4), whereas another mutant altering DLQP region (which is not involved in CHC interaction, Figure 7D, lane 5) was still able to rescue the endocytosis (Figure 7H, lane 5). Furthermore, mouse Tom1L1 mutants equivalent to human Tom1L1/Y460F (lane 6) or Tom1L1/Y392F (lane 7) also failed to rescue the defect of EGFR endocytosis in the knockdown cells. These results suggest that interactions with clathrin and EGFR are both important for Tom1L1 to mediate endocytosis of EGFR in response to EGF stimulation.

Discussion

Although AP-2 adaptor complex is the major adaptor important for endocytosis of diverse surface proteins, several independent lines of evidence suggest that endocytosis of EGFR is primarily mediated by an AP-2-independent, but clathrin-dependent process (Nesterov *et al*, 1999; Conner and Schmid, 2003; Motley *et al*, 2003; Traub, 2003; Lakadamyali *et al*, 2006). Consistent with these studies, knockdown of CHC inhibited endocytosis of EGFR to levels comparable to those when Tom1L1 was knocked down,

whereas knockdown of AP-2 had little effect on endocytosis of EGFR, although it potently inhibited endocytosis of TfR (Supplementary Figure S6). These studies suggest that additional or alterative adaptor (s) is responsible for bridging EGFR with the endocytic machinery. Our studies suggest that Tom1L1 is a regulated adaptor for EGFR endocytosis. First, Tom1L1 is transiently associated with activated EGFR during the very early events of EGF stimulation. This interaction depends on transient Tyr-phosphorylation of Tom1L1 through EGFR-activated Src kinases and is likely to be mediated by



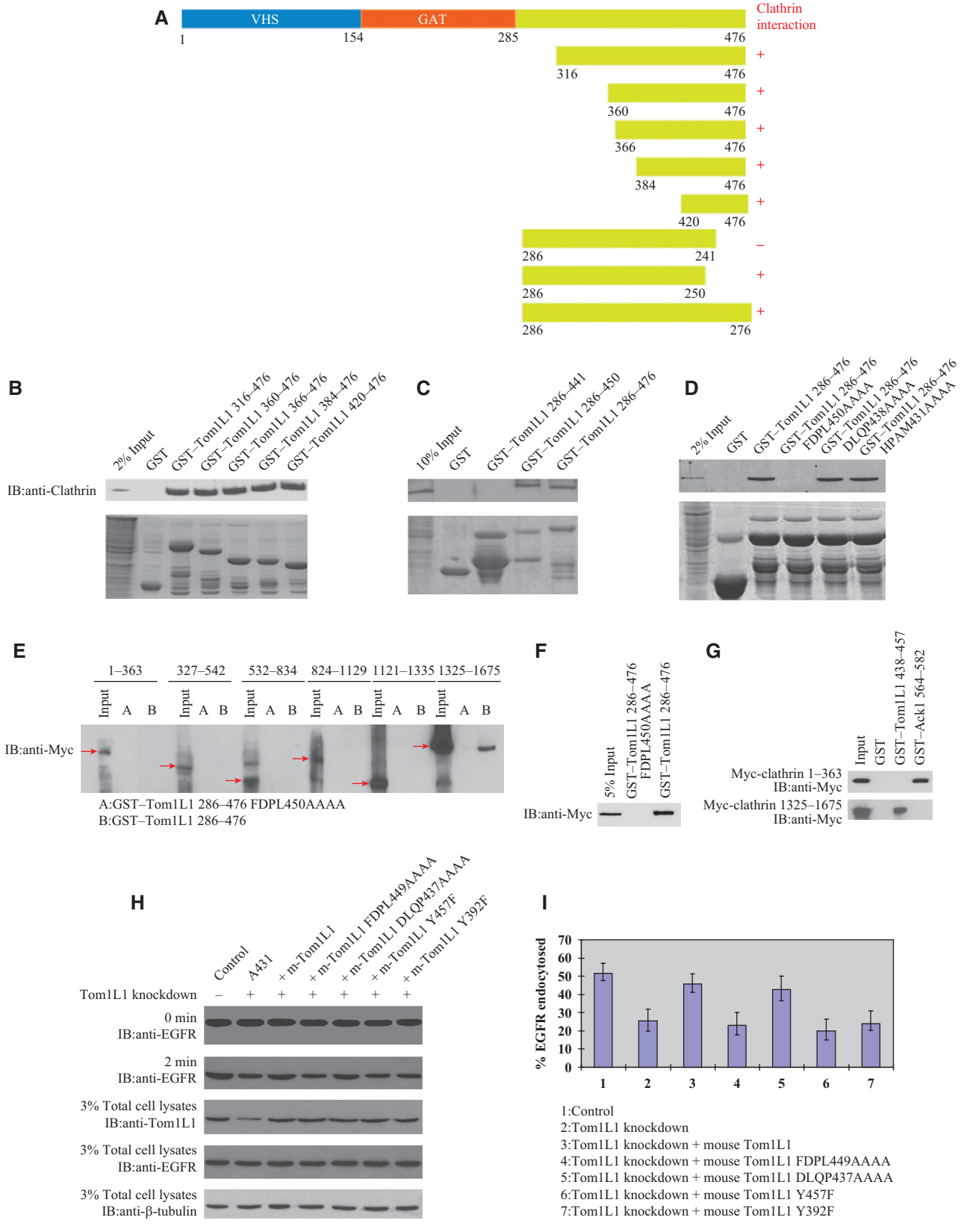
Grb2 and Shc as bridging proteins. Second, Tom1L1 harbours a novel clathrin-interacting motif that is not only essential for interaction with clathrin, but also important for exogenous Tom1L1 to functionally rescue endocytosis of EGFR in Tom1L1 knockdown cells. Third, on EGFR activation, Tom1L1 was rapidly recruited from the cytoplasm to the plasma membrane and redistributed with EGFR into the early endosome. In support of biochemical and morphological results, Tom1L1 is important for EGFR endocytosis. First, the extent of EGFR endocytosis correlates well with Tyr-phosphorylation of EGFR and Tom1L1, and interaction of Tom1L1 with EGFR when cells were treated with different concentrations of EGF (Supplementary Figure S7A). Second, mutants of Tom1L1 incapable of interaction with EGFR because of a defect in Tyr-phosphorylation (Tom1L1/Y460F) or interaction with Grb2 (Tom1L/Y392F) behaved similar to dominant-negative mutants. Third, RNAi-mediated knockdown of endogenous Tom1L1, but not Tom1 or Tom1L2, inhibited EGFR endocytosis. Importantly, exogenous Tom1L1, but not Tom1, was able to significantly rescue the endocytosis defect of EGFR in Tom1L1 knockdown cells. This system also allowed us to show that the clathrin-interacting motif and the motifs responsible for interaction with activated EGFR are important for exogenous Tom1L1 to rescue the endocytosis of EGFR. This correlation between Tom1L1 and EGFR interaction and a crucial function for Tom1L1 in endocytosis provides mechanistic insights into the function of Tom1L1 as a regulated adaptor mediating endocytosis of EGFR. Consistent with a function of Src, Grb2 and Shc in mediating interaction of Tyr-phosphorylated Tom1L1 with activated EGFR, treatment of the Src kinase inhibitor PP1 or knockdown of Grb2 or Shc significantly inhibited endocytosis of EGFR (Wilde *et al*, 1999; Jiang *et al*, 2003; Huang and Sorkin, 2005) (Supplementary Figure S7B and C), which is further supported by the robust inhibition of Tom1L1–EGFR interaction (Figure 2D) and EGFR endocytosis (Supplementary Figure S7C) when both Grb2 and Shc were knocked down. The reported association of Grb2 with clathrin-coated buds/vesicles responsible for EGFR endocytosis (Johannessen *et al*, 2006) and the interaction between Grb2 SH3 domain and N-terminal pro-rich region of Shc (Khanday *et al*, 2006) also supports our hypothesis. The fact that GGAs, other VHS domain proteins, act as adaptors in mediating sorting of M6PR at the TGN (Bonifacino, 2004) is also consistent with this conclusion. These results allow us to propose a working model for Tom1L1 to act as a regulated adaptor in mediating EGF-stimulated endocytosis of EGFR (Figure 7J).

It is noteworthy that, in response to EGF stimulation, Tom1L1 is initially associated with the plasma membrane and subsequently enriched in the early endosome with EGFR during endocytosis. The latter occurs 20–30 min after EGF stimulation, and by then Y460 of Tom1L1 is already dephosphorylated. It thus seems likely that the early endosome-associated Tom1L1 may engage in interactions with other proteins such as Hrs and TSG101; and this endosomal Tom1L1 may participate in the efficient segregation of EGFR in the maturing endosome to form the MVB (the late endosome) for subsequent delivery to the lysosome (Futter *et al*, 1996; Puertollano, 2005; Lakadamyali *et al*, 2006; Razi and Futter, 2006). In this way, phosphotyrosine-mediated recruitment of Tom1L1 to activated EGFR on the plasma membrane not only triggers internalization, but may also prime Tom1L1 to stay onto the early endosome for subsequent interaction with the sorting machinery for efficient segregation of EGFR into the MVB. Further experiments are needed to examine this hypothesis.

One interesting issue to be resolved by future experiments is the relationship between Tom1L1 and c-Cbl during EGFR endocytosis. c-Cbl is known to be an E3-ubiquitinating enzyme for attaching multiple mono-ubiquitin moieties to the cytoplasmic domain of EGFR, a modification involved in endocytosis and subsequent sorting of internalized receptor to MVB (Rubin *et al*, 2005; Citri and Yarden, 2006). Consistent with a function of c-Cbl in endocytosis, knockdown of Cbl also inhibited endocytosis of EGFR (Supplementary Figure S6). Although c-Cbl is important in mediating EGFR endocytosis by mono-ubiquitinating multiple sites in the cytoplasmic tail of EGFR, RNAi of individual ubiquitin-interacting proteins such as EPS15, ESP15R or Epsin has little

Figure 6 Knockdown of Tom1L1 delays EGF-induced degradation and endocytosis of EGFR. (**A**) A431 cells transfected with control non-targeting siRNA (lane 1), GAPDH-targeting siRNA (lane 2) or Tom1L1-targeting siRNA (lane 3) were incubated with EGF (100 ng/ml) at 37°C for 0 or 3 h. Cell lysates were analysed by immunoblot to detect EGFR at 0 h (upper panel) or 3 h (second panel), Tom1L1 at 3 h (third panel) and GAPDH at 3 h (bottom panel). (**B**) The results from three independent experiments as exampled in (A) were quantified by densitometric analysis for EGFR levels at 0 and 3 h. Each column represents the percentage of EGFR detected at 3 h relative to 0 h, normalized against β-tubulin (not shown). The mean ± s.d. are shown. (**C**) A431 cells transfected with control non-targeting siRNA (upper panel), GAPDH-targeting siRNA (middle panel) or Tom1L1-targeting siRNA (bottom panel) were surface biotinylated with sulfo-NHS-SS-biotin. After incubation with EGF (100 ng/ml) at 37°C for the indicated period of time, surface-exposed biotin was removed by stripping in reducing buffer. Biotinylated proteins were recovered and analysed by immunoblot to detect EGFR. (**D**) The results from three independent experiments exampled in (C) were quantified by densitometric analysis. Each signal is normalized against the level of β-tubulin detected at each time point (not shown). Values are expressed as percentage of signal at each time point relative to signal detected at 0 min (100%). The mean ± s.d. are shown. (**E**) A431 cells transfected with indicated siRNA were then infected with retrovirus expressing RNAi-resistant mouse Tom1L1 (lane 3) or human Tom1 (lane 4) to examine the ability to restore EGFR internalization. The amount of biotinylated EGFR at 0 min is shown in the first panel, whereas the amount of biotinylated EGFR endocytosed after 2 min is shown in the second panel. The cell lysates were also analysed as indicated (the bottom panels). (**F**) The results from three independent experiments exampled in (E) were quantified by densitometric analysis. Each signal is normalized against the level of β-tubulin detected at 2 min. Values are expressed as ratio of signal at 2 min to that detected at 0 min (100%). The mean ± s.d. are shown. (**G**) A431 cells were transfected with control non-targeting siRNA or siRNA-targeting Tom1, Tom1L1 or Tom1L2 as indicated. Starved cells were surface biotinylated. Cells were stimulated with EGF (100 ng/ml) at 37°C for 2 min to induce endocytosis of EGFR. Biotinylated and endocytosed EGFR at 2 min of EGF stimulation was recovered and then analysed by immunoblot (second panel). The total amount of biotinylated EGFR at 0 min (first panel) was also detected. Starting cell extracts were also analysed as loading controls (the bottom 4 panels). (**H**) The amount of total biotinylated EGFR at 0 min as well as those endocytosed after 2 min were quantified by densitometric analysis and normalized against the levels of β-tubulin. The average of three independent experiments expressed as the percentage of the amount of endocytosed receptor at 2 min relative to the total amount at 0 min is shown as mean ± s.d.

effect on EGFR endocytosis (Huang *et al*, 2004). As EGFR is ubiquitinated for a prolonged period of time from 2 min onwards until 120 min after EGF stimulation (Shtiegman *et al*, 2007; Stern *et al*, 2007), whereas Tom1L1 is only transiently associated with Tyr-phosphorylated EGFR during 2–10 min period of EGF stimulation, interaction of Tom1L1 with EGFR correlated with Tyr-phosphorylation rather than ubiquitination of EGFR (Shtiegman *et al*, 2007; Stern *et al*, 2007). On this note, Tom1L1 may act independently of c-Cbl for interaction with EGFR. A recent study has



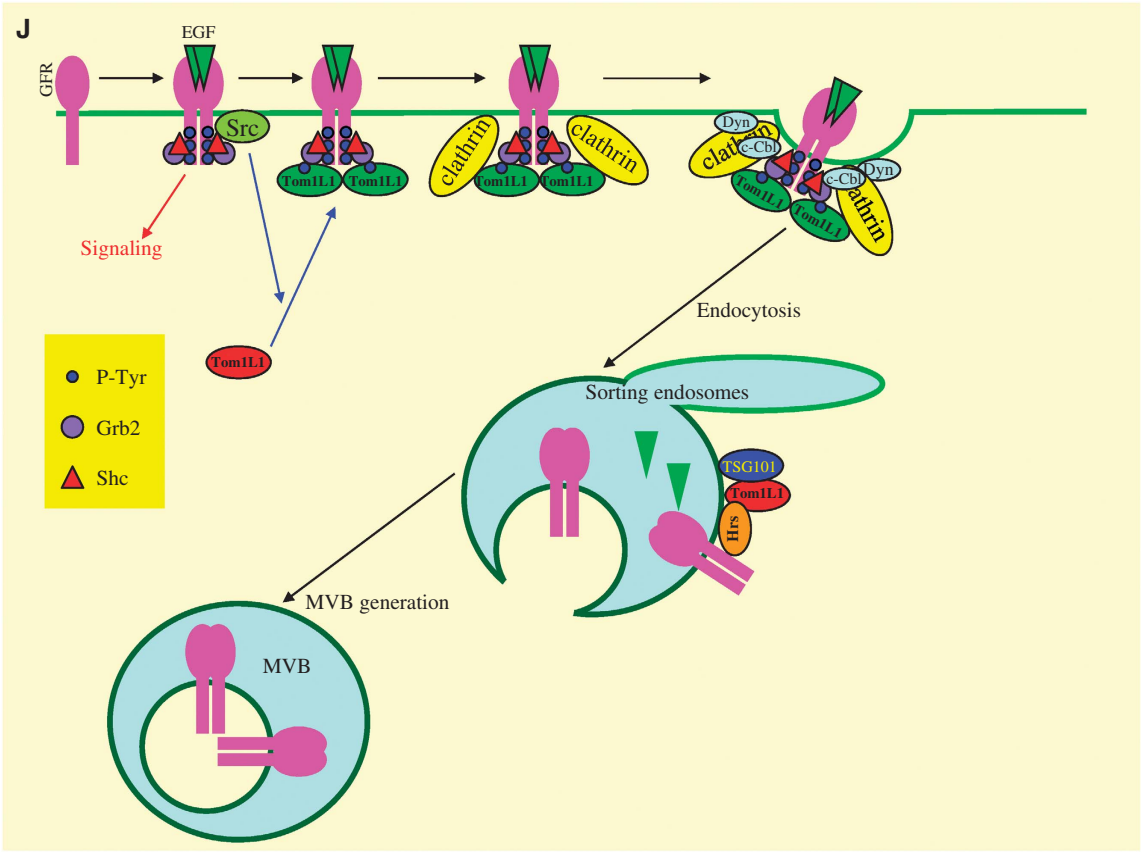


Figure 7 Continued.

Figure 7 Tom1L1 contains a novel clathrin-binding motif important for endocytosis of EGFR. (**A**) Schematic diagram of various C-terminal fragments of Tom1L1 expressed as GST-fusion proteins and their ability to interact with the CHC. (**B**–**D**) Various C-terminal fragments of Tom1L1 in the form of GST-fusion proteins were immobilized onto glutathione-sepharose beads. Cytosol derived from A431 cells was incubated with these beads and the proteins retained by the beads were resolved by SDS–PAGE followed by immunoblot to detect CHC (upper panel). GST and GST-fusion proteins were stained by Coomassie blue (bottom panel). Aliquots of cytosol were also analysed as loading control. (**E**) Various fragments of CHC in the form of Myc-tagged polypeptides as indicated (the fragments of expected size were indicated by red arrow heads) were expressed in the TNT system. A measure of 50 μl of each of the translation reactions were incubated with immobilized GST–Tom1L1(286–476) FDPL450AAAA or GST–Tom1L1(286–476), respectively, and the proteins retained by the beads were resolved by SDS–PAGE followed by immunoblot to detect the Myc-tagged fragments. Only C-terminal fragment (residues 1325–1675) was retained by GST–Tom1L1 (286–476), but not by the mutant FDPL450AAAA. (**F**) Myc-tagged C-terminal fragment (residues 1325–1675) of CHC was expressed in the TNT system and then purified by immunoprecipitation. The eluted proteins were incubated with immobilized GST-fusion proteins and the retained proteins were analysed by immunoblot to detect the Myc-tagged fragment of CHC. GST–Tom1L1(286–476), but not GST–Tom1L1(286–476)FPDL450AAAA was able to retain the CHC fragment. (**G**) Myc-tagged N-terminal (1–363) or C-terminal (1325–1676) domain of CHC expressed in the TNT system was incubated with immobilized GST, GST–Tom1L1(438–457) or GST–Ack1(564–582), respectively, and the proteins retained by the beads were resolved by SDS–PAGE followed by immunoblot to detect the Myc-tagged fragments of CHC. (**H**) A431 cells were transfected with control non-targeting siRNA (lane 1) or Tom1L1-targeting siRNA (other lanes). The cells were either not infected (lanes 1–2) or infected with retrovirus to express RNAi-resistant mouse Tom1L1 (lane 3) or the indicated mutants (lanes 4–7) to determine their ability to rescue EGFR internalization. The amount of biotinylated EGFR at 0 min is shown in the first panel, whereas the amount of biotinylated EGFR endocytosed after 2 min is shown in the second panel. As shown, the reduced EGFR endocytosis caused by RNAi of Tom1L1 (lane 2) can be restored significantly by exogenous mouse Tom1L1 (lane 3), mouse Tom1L1/DLQP437AAAA (lane 5), but not mouse Tom1L1/FDPL449AAA defective in clathrin-binding (lane 4), mouse Tom1L1/Y457F (which is equivalent to human Tom1L1–Y460F defective in Tyr-phosphorylation) (lane 6) or Tom1L1/Y392F (which is defective in interaction with Grb2) (lane 7). (**I**) The results from three independent experiments were quantified. Each signal is normalized against the level of β-tubulin detected at 2 min. Values are expressed as percentage of signal at 2 min relative to that detected at 0 min (100%). The mean ± s.d. are shown. (**J**) A working model for Tom1L1 to act as a regulated adaptor mediating EGF-stimulated endocytosis of EGFR. On EGF stimulation, EGFR dimerizes, leading to the activation of its cytoplasmic kinase domain and Tyr-phosphorylation at multiple sites, which then serve as docking sites for various signalling proteins such as Shc/Grb2 and Src kinases; activated Src family kinases phosphorylate Y460 of Tom1L1, causing a transient interaction of pTom1L1 with the activated EGFR through Grb2/Shc. By interacting with clathrin, pTom1L1 can bring endocytic machinery to mediate the segregation of activated EGFR into clathrin-coated structures for endocytosis. Other proteins such as dynamin and c-Cbl may be independently recruited to complete the endocytosis. After endocytosis, pTom1L1 becomes dephosphorylated and the dephosphorylated Tom1L1 is retained on the early endosome probably through its interaction with Hrs and TSG101 (Puertollano, 2005), in which it may potentially facilitate the sorting of EGFR into the MVB.

shown that ubiqutination-deficient mutant EGFR is endocytosed normally, but severely impaired in degradation by the lysosome because of an arrest in the early endosome. These results suggest that c-Cbl-mediated ubiquitination of EGFR may primarily regulate endosomal sorting to the MVB/lysosome and another mechanism may account for c-Cbl participation in endocytosis (Huang *et al*, 2006). Dual recognition of EGFR by Tom1L1 and c-Cbl is likely to be important in mediating overlapping aspects of EGFR traffic in which Tom1L1 acts primarily in endocytosis, whereas c-Cbl-mediated ubiquitination acts mainly in the endosomal sorting of EGFR (Clague and Urbe, 2006; Huang *et al*, 2006; Su *et al*, 2007).

It was reported that, in HeLa cells, there exist two different trafficking routes for EGFR internalization depending on the dose of EGF (Sigismund *et al*, 2005). At low EGF doses, EGFR endocytosis is mediated by clathrin-dependent pathway, whereas high doses of EGF trigger EGFR internalization through both clathrin-dependent and clathrin-independent pathways. In our study, we have observed that endocytosis of EGFR in A431 cells correlates well with the dose-dependent activation of EGFR, Tyr-phosphorylation of Tom1L1 and interaction of Tom1L1 with EGFR in that optimal endocytosis of EGFR was observed at 15–20 ng/ml of EGF or higher (Supplementary Figure S7A, lanes 4–7). Lower dose of EGF stimulated less endocytosis (Supplementary Figure S7A, lanes 1–3), correlating with partial Tyr-phosphorylation of EGFR and Tom1L1, and less interaction of Tom1L1 with EGFR. The endocytosis we were measuring thus correlates well with activation of EGFR, Tyr-phosphorylation of Tom1L1 and interaction of Tyr-phosphorylated Tom1L1 with activated EGFR. Consistently, endocytosis of EGFR induced by 15 ng/ml of EGF was similarly inhibited by Tom1L1/Y460 (Supplementary Figure S8A, second panel) to a level comparable to the inhibition observed in cells stimulated with 100 ng/ml of EGF (Supplementary Figure S8A, third panel). In this context, EGFR signalling and trafficking in A431 cells (Figure 2F, Supplementary Figures 7A and S8A) are somehow different from those in Hela cells. Two recent studies showed that endocytosis of EGFR is primarily mediated by clathrin-dependent pathway, even in the presence of higher concentrations of EGF (up to 60 ng/ml in HeLa cells or 200 ng/ml in BSC-1 cells) (Kazazic *et al*, 2006; Lakadamyali *et al*, 2006). Furthermore, Tom1L1/Y460 or Tom1L1 knockdown also inhibited endocytosis of EGFR triggered by 1.5 ng/ml of EGF in HeLa cells (Supplementary Figure S8B–D). As an early study indicates that the normal function of AP2 in EGFR endocytosis may be bypassed by experimental manipulation of incubating cells with EGF first at 4°C followed by endocytosis at 37°C (Huang *et al*, 2004), we have also performed experiments in which both binding and endocytosis were carried out at 37°C. The results from these experiments also support a function of Tom1L1 in endocytosis of EGFR in response to EGF (Supplementary Figure S8C and D). Consistent with these observations, endocytosis of EGFR elicited by 100 ng/ml of EGF in A431 was potently suppressed by knockdown of CHC (Supplementary Figure S6). These results, taken together, suggest that Tom1L1 has a general function in EGF-stimulated endocytosis of EGFR, regardless of experimental conditions or cell types.

Materials and methods

These were described as part of the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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