# An endosomally localized isoform of Eps15 interacts with Hrs to mediate degradation of epidermal growth factor receptor

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Down-regulation of activated and ubiquitinated growth factor (GF) receptors by endocytosis and subsequent lysosomal degradation ensures attenuation of GF signaling. The ubiquitin-binding adaptor protein Eps15 (epidermal growth factor receptor [EGFR] pathway substrate 15) functions in endocytosis of such receptors. Here, we identify an Eps15 isoform, Eps15b, and demonstrate its expression in human cells and conservation across vertebrate species. Although both Eps15 and Eps15b interact with the endosomal sorting protein Hrs (hepatocyte growth factor–regulated tyrosine kinase substrate) in vitro, we find that Hrs specifically binds Eps15b in vivo (whereas adaptor protein 2 preferentially interacts with Eps15). Although Eps15 mainly localizes to clathrin-coated pits at the plasma membrane, Eps15b localizes to Hrs-positive microdomains on endosomes. Eps15b overexpression, similarly to Hrs overexpression, inhibits ligand-mediated degradation of EGFR, whereas Eps15 is without effect. Similarly, depletion of Eps15b but not Eps15 delays degradation and promotes recycling of EGFR. These results indicate that Eps15b is an endosomally localized isoform of Eps15 that is present in the Hrs complex via direct Hrs interaction and important for the sorting function of this complex.

## Introduction

Growth factor signaling mediates cell proliferation and differentiation and is a tightly regulated process. Both activation and inactivation of signaling cascades are controlled by multiple mechanisms to ensure a correct cellular response level to a given stimulus. Endocytosis and endosomal sorting of growth factor receptors is a major mechanism for such inactivation by internalizing and sorting activated receptors into intraluminal vesicles of multivesicular bodies. This renders the receptors inaccessible for peripheral signaling components and destines them for degradation in lysosomes (Felder et al., 1990).

Receptors due to follow the pathway toward lysosomal degradation are distinguished from those destined for other transport routes by the covalent addition of the small protein ubiquitin to the cytosolic region of the receptor, extensively studied in the case of the EGF receptor (EGFR). This sorting event occurs at early endosomes, where a protein complex containing the hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs) recruits ubiquitinated receptors to clathrin-coated microdomains (Raiborg et al., 2002). In concert with the evolutionarily conserved endosomal sorting complex required for transport (ESCRT) machinery, which consists of the ESCRT-I, -II and -III complexes, Hrs mediates the formation of intraluminal endosomal vesicles and the accumulation of receptors in these vesicles (Katzmann et al., 2001; Babst et al., 2002a,b; Bache et al., 2003). The initial recruitment of a ubiquitinated receptor to the sorting machinery is thought to be mediated by direct interactions between the ubiquitin moiety and a ubiquitin-interacting motif (UIM) in Hrs (Bilodeau et al., 2002; Raiborg et al., 2002; Hirano et al., 2006). Hrs is localized to early endosomes via an interaction with phosphatidylinositol-3-phosphate (Raiborg et al., 2001b). In addition to recruiting ubiquitinated cargo to early endosomes, Hrs is required for endosomal localization of several components of the sorting machinery, including signal-transducing adaptor molecule (STAM), ESCRT-I, and clathrin. Depleting cells of Hrs results in impaired degradation of activated EGFR, which illustrates the essential role of Hrs in the process of endosomal protein sorting (Bache et al., 2003).

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Abbreviations used in this paper: AP2, adaptor protein 2; EEA1, early endosomal antigen 1; EGFR, EGF receptor; EH, Eps15 homology; Eps15, EGFR pathway substrate 15; ESCRT, endosomal sorting complex required for transport; Hrs, hepatocyte growth factor–regulated tyrosine kinase substrate; STAM, signal-transducing adaptor molecule; TSS, transcriptional start site, UIM, ubiquitin-interacting motif.


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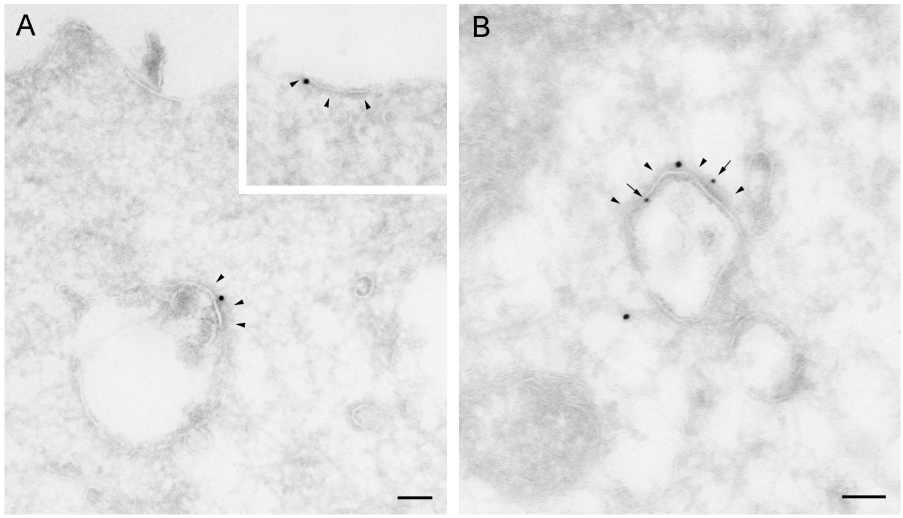


Figure 1. **An Eps15-related protein is present in bilayered coats on endosomes.** (A) HEp-2 cells incubated with EGF on ice and chased for 15 min at 37°C were prepared for immunoelectron microscopy and labeled using an antibody recognizing the C terminus of Eps15. (B) HeLa cells incubated with EGF on ice and chased for 15 min at 37°C were prepared for immunoelectron microscopy and labeled for Eps15 (15 nm gold) followed by labeling for Hrs (10 nm gold, arrows). The labeling for Eps15 localized to bilayered coats on endosome-like compartments as well as to the rim of coated pits at the plasma membrane (inset in A). The two micrographs shown in A are from the same cell. Coats are indicated by arrowheads. Bars, 100 nm.

In addition to Hrs and STAM, the endocytic adaptor protein Eps15 has been reported to participate in the Hrs complex via a direct interaction with Hrs (Bean et al., 2000; Bache et al., 2003). The role of Eps15 in the more upstream process of endocytosis has been extensively studied, establishing Eps15 as a part of the complex protein interaction network present at clathrin-coated pits (Salcini et al., 1999; Conner and Schmid, 2003; Sigismund et al., 2005). Eps15 is constitutively associated with the clathrin-coated pit adaptor protein 2 (AP2) complex and is localized to the cytosol and the rims of clathrin-coated pits (Benmerah et al., 1995; Tebar et al., 1996). The Eps15 protein consists of several functional domains (Fazioli et al., 1993). The three N-terminal Eps15 homology (EH) domains are protein–protein interaction modules responsible for the majority of the interactions Eps15 form with other proteins (Wong et al., 1995; Salcini et al., 1997; Polo et al., 2003). These domains further mediate the ability of Eps15 to stimulate clathrin assembly at emerging clathrin coated pits through interacting with the adaptor protein AP180 and are furthermore required for the recruitment of Eps15 to clathrin-coated pits (Benmerah et al., 1999; Morgan et al., 2003). The intermediate coiled-coil domain serves as the interacting surface for the constitutive oligomerization of Eps15 and can additionally interact with Hrs (Tebar et al., 1997; Bean et al., 2000). Toward the C-terminal end of the protein, a series of DPF repeats constitute the binding domain needed for the association with AP2, and this domain is also capable of interacting with Hrs (Benmerah et al., 1996; Bean et al., 2000). The C terminus of Eps15 contains two UIMs, the last of which is indispensable for the ability of Eps15 to bind ubiquitin, whereas both UIMs are necessary for monoubiquitination of Eps15 itself (Polo et al., 2002). Although the bulk of Eps15 resides in the cytosol or at clathrin-coated pits, a small pool of Eps15 has been reported to localize to early endosomes. The functional significance of the endosomally localized Eps15 is unknown. Here, we identify a novel isoform of Eps15, Eps15b, and show evidence that this protein localizes to endosomes and participates with Hrs in endosomal sorting of endocytosed growth factor receptors.

## Results

### An Eps15-related protein is found in bilayered clathrin coats on endosomes

Previous studies have shown that Hrs resides in a characteristic "bilayered" clathrin coat on early endosomes, where it is involved in retention and lysosomal sorting of ubiquitinated membrane proteins (Raiborg et al., 2002; Sachse et al., 2002). Clathrin appears to retain Hrs molecules in restricted microdomains, thereby increasing their local concentration beyond a critical value required for cargo sorting through low-affinity interactions (Raiborg et al., 2006). Confocal microscopy has shown that an anti-Eps15 immunoreactive protein colocalizes with Hrs on endosomes (Bache et al., 2003) but the resolution of the light microscope is insufficient to establish whether Eps15 is present in the bilayered clathrin coat. To address this issue, we studied HEp-2 and HeLa cells by immunoelectron microscopy using an antibody against the C-terminal part of Eps15 followed by protein A conjugated to colloidal gold. The endosomal labeling with anti-Eps15 was low and we rarely detected more than one immunogold particle per endosome. However, the labeling was highly specific, and we consistently detected anti-Eps15 labeling within the bilayered coat of endosomes (Fig. 1 A). Double labeling with anti-Eps15 and anti-Hrs showed that the bilayered Eps15-positive coat contained Hrs as well (Fig. 1 B). As reported previously (Raiborg et al., 2002, Sachse et al., 2002), this coat was morphologically distinct from clathrin-coated pits at the plasma membrane, in which we could also detect Eps15 labeling (Fig. 1 A, inset). These results indicate that an Eps15-related protein is found in the bilayered clathrin coats on endosomes.

### Eps15b is an isoform of Eps15

The finding that Eps15 proteins localize to two distinct membranes begged the question of whether the form of Eps15 residing in bilayered clathrin coats on endosomes might be distinct from the canonical clathrin-coated pit-associated form. Indeed, in their initial characterization of Eps15, Fazioli et al. (1993) identified several protein species recognized by Eps15 antibodies and introduced the idea of possible isoforms of Eps15.

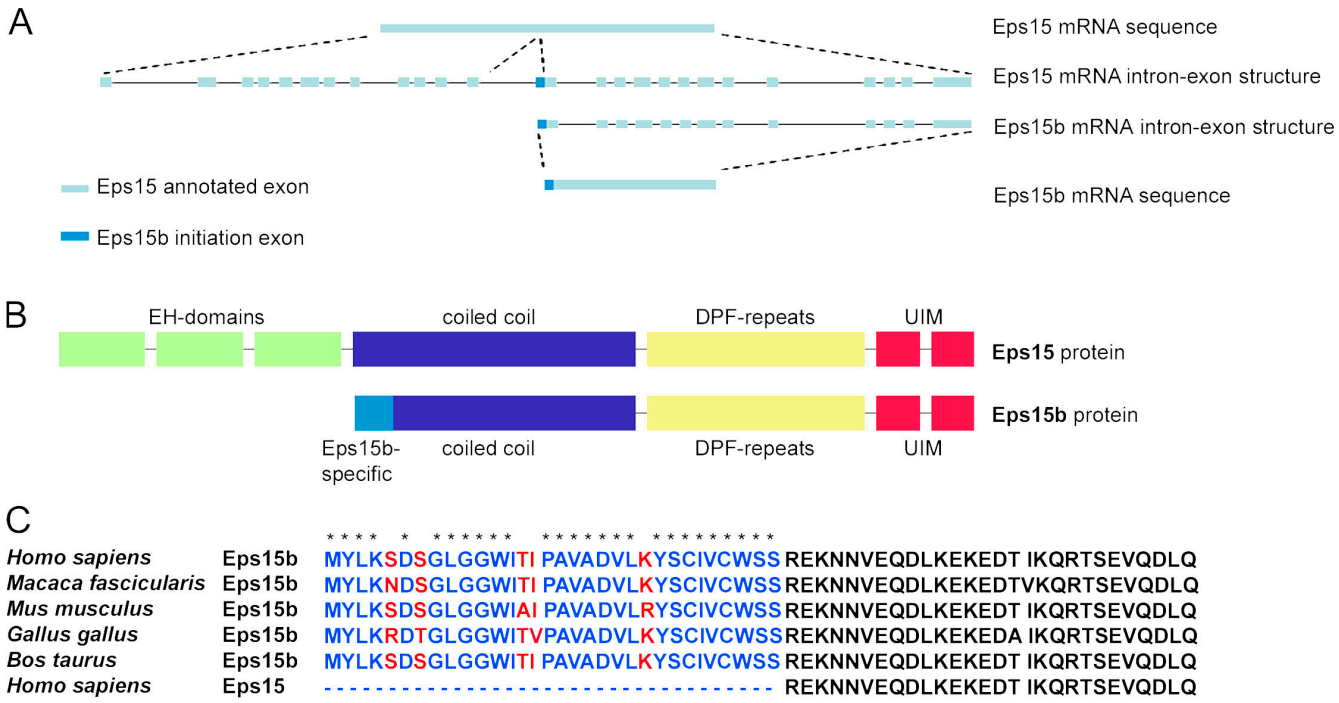


Figure 2. **Eps15b is an isoform of Eps15.** (A) The *EPS15* gene contains 25 annotated exons. Eps15 is produced from exons 1–25, whereas the Eps15b isoform originates from a transcriptional initiation in intron 12 continuing through exons 13–25. (B) The Eps15 protein contains several functional protein domains: three EH domains, a coiled-coil region, a DPF repeat domain, and two consecutive UIMs. The Eps15b isoform lacks the three EH domains and contains a short unique N-terminal region. The remaining part of the protein is identical to Eps15 and contains major parts of the coiled coil region in addition to the DPF repeat domain and the UIM motives. (C) Multiple sequence alignment of the predicted protein sequences for the putative Eps15b homologues in *Homo sapiens* (BX647676), *Macaca fascicularis* (AB172746), *Mus musculus* (BAC38796), *Gallus gallus* (CR407454), and *Bos taurus* (DV911715) compared with Eps15 from *H. sapiens*. The sequences in blue and red represent the N-terminal 32 Eps15b-specific sequences (conserved residues are shown in blue and unconserved residues in red), whereas the sequence in black indicates where Eps15b aligns with the Eps15 sequence. The asterisks indicate fully conserved amino acid residues in the Eps15b-specific region.

Through searches in public databases, we identified a putative isoform of Eps15 on the basis of a full-length cDNA sequence (GenBank/EMBL/DDBJ accession no. BX647676; isolated from a human colon endothelial primary cell culture) and named it Eps15b (Fig. 2 A). The *EPS15B* sequence comprises exons 13–25 of the *EPS15* gene and upstream 245 additional nucleotides. These 245 nucleotides align with the 3′ end of intron 12 according to the genomic sequence of *EPS15* and we consequently defined this region of intron 12 as an alternative transcriptional initiation exon. These 245 nucleotides form the 5′ end of the *EPS15B* isoform and suggest that an alternative promoter and transcriptional start site (TSS) exists in intron 12. The DataBase of Human Transcription Start Sites (Suzuki et al., 2004), containing large-scale information on TSSs based on experimentally determined TSSs of human genes, identifies several TSSs in the ±30 nucleotide vicinity of the *EPS15B* start site, which suggests that this region is an active site of transcriptional initiation.

The *EPS15B* sequence contains an ATG translational start site with an open reading frame predicted to produce a protein of 582 amino acids (Fig. 2 B). Out of these 582 residues, the first 32 are unique for Eps15b and, as the ATG in the alternative initiation exon lies in frame with the Eps15 sequence starting at exon 13, it is followed by the 550 most C-terminal residues of Eps15. The resulting Eps15b protein is truncated in the N terminus relative to Eps15 and lacks the 346 first amino acids of Eps15 resulting from translation of exon 1–12. Consequently, the Eps15b form lacks the three N-terminal EH domains required for the recruitment of Eps15 to clathrin-coated pits (Benmerah et al., 1999). The N-terminal Eps15b-specific sequence served to identify EST clones corresponding to the Eps15b isoform isolated from different human tissues, such as brain (GenBank/EMBL/DDBJ accession no. DA801048) and embryonic stem cells (GenBank/EMBL/DDBJ accession no. CN346061). The EST clones identified differ slightly with respect to the 5′ end, and further analyses are required to verify the exact transcriptional start site. Quantitative real-time PCR using Eps15b-specific primers revealed that Eps15b is expressed in HeLa cells (see the following paragraph), and together these results indicate that the Eps15b isoform is expressed in a variety of human cell types. The Eps15b unique sequence further enabled the identification of related cDNA clones representing putative homologues in other animal species, indicating that the isoform is conserved across species. The predicted N-terminal amino acid sequences of these putative Eps15b homologues displayed 84% conservation between humans and birds in the Eps15b-specific region, indicating a strong conservation pressure (Fig. 2 C).

To compare Eps15b with the putative Eps15 isoforms previously observed, we translated the protein in vitro. Western blot analysis revealed that Eps15b migrates at ∼120 kD (Fig. 3 A). In line with this observation, Fazioli et al. (1993)

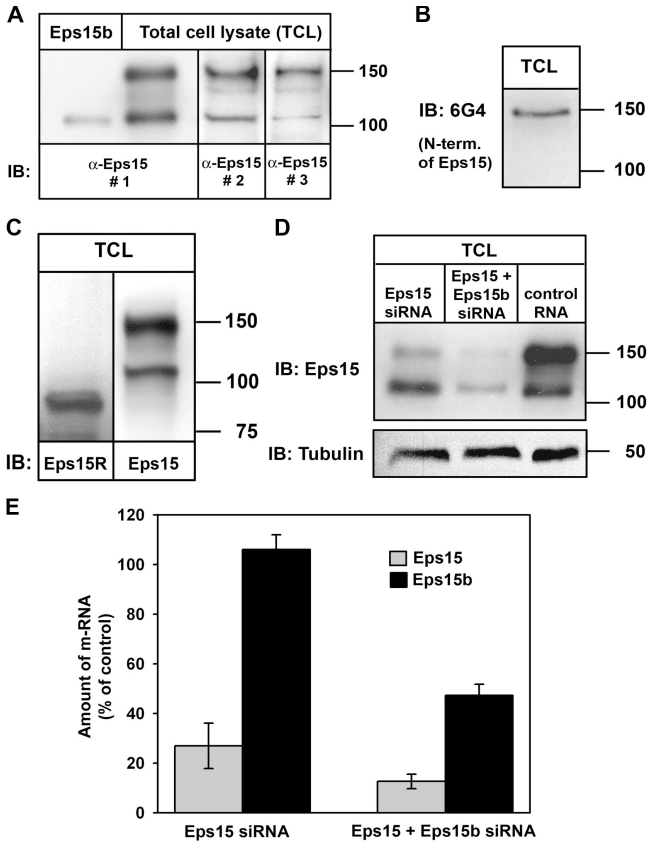


Figure 3. **Eps15b migrates at 120 kD and is expressed in HeLa and HEp-2 cells.** (A) In vitro translated Eps15b was compared with endogenous anti-Eps15 immunoreactive protein species from a HeLa cell lysate by SDS-PAGE followed by immunoblotting using anti-Eps15 from Santa Cruz Biotechnology. The total cell lysate pattern was reconfirmed with two additional antibodies generated against the C terminus of Eps15, anti-Eps15 No. 2 from Covenance, and anti-Eps15 No. 3 from Abcam. (B) HeLa cells were lysed and analyzed by SDS-PAGE and immunoblotting with the 6G4 antibody raised against the N-terminal region of Eps15. (C) Eps15 and Eps15R immunoreactive proteins were compared by SDS-PAGE and immunoblotting using anti-Eps15R followed by stripping of the membrane and reprobing with anti-Eps15. (D) HeLa cells were transfected with siRNA oligos targeting Eps15, Eps15, and Eps15b, or a nontargeting control RNA duplex as described in Materials and methods. The level of knockdown was assessed by Western analysis using anti-Eps15 and anti-tubulin. Numbers to the right of gel blots indicate molecular mass in kD. (E) The level of knockdown was additionally investigated by real-time PCR using Eps15- and Eps15b-specific primers.

registered a protein recognized by an Eps15 antibody in NIH 3T3 cells migrating at 120 kD in addition to the full-length Eps15 protein migrating at 150 kD. We confirmed this pattern in HEp-2 and HeLa cells; and by Western blot analysis of cell lysates using three different antibodies generated against the C terminus of Eps15, we observed two immunoreactive proteins: one major band at 150 kD corresponding to Eps15 and a second weaker band at 120 kD (Fig. 3 A). To further explore the possibility that this endogenous 120-kD protein could correspond to the Eps15b isoform, we used an antibody raised against the N-terminal EH domains of Eps15, which are absent in the Eps15b isoform. This antibody recognized the 150-kD Eps15 protein but not the 120-kD protein in HeLa cells (Fig. 3 B). To rule out that the 120-kD immunoreactive protein was a result of antibody cross-reactivity with the related Eps15R, we compared the migration of the Eps15 and Eps15R immunoreactive proteins (Fig. 3 C). In our hands, the major Eps15R reactive species migrated close to the calculated $M_r$ of 99 kD for Eps15R, whereas previous studies in HeLa cells have reported an apparent $M_r$ of 108 for Eps15R (Coda et al., 1998). We believe this discrepancy is related to our use of a new SDS-PAGE system that yields an apparent faster migration for certain proteins. Importantly, the faster migration of Eps15R compared with the Eps15-reactive species excludes the possibility that the latter represents Eps15R.

Finally, we tried to deplete the cells of Eps15 and Eps15b by siRNA-mediated knockdown (Fig. 3 D). By using an siRNA oligonucleotide directed against the N-terminal EH domains of Eps15, we observed a specific knockdown of the 150-kD protein, whereas an oligonucleotide directed against the C-terminal region common for Eps15 and Eps15b significantly reduced both the 150- and 120-kD α-Eps15 immunoreactive proteins. These knockdown results were reconfirmed on the RNA level by quantitative real-time PCR using Eps15- and Eps15b-specific primers (Fig. 3 E). There was, however, a clear difference in efficiency of the siRNA oligonucleotides; although 80–90% of Eps15 was knocked down, the Eps15b expression was only reduced by ∼50%. This was repeatedly observed both at the protein and the RNA level and may be caused by differences in target accessibility for the siRNA oligonucleotide because of differential secondary structures of the Eps15 and Eps15b mRNA. Our attempts to specifically knock down the Eps15b isoform were unsuccessful, as the short Eps15b-specific sequence only allowed the design of suboptimal siRNA oligonucleotides. Collectively, our data suggest that the endogenous 120-kD protein recognized by anti-Eps15 corresponds to the isoform Eps15b.

### Eps15b forms a tight complex with Hrs

Eps15 has been proposed to exist in complex with Hrs on early endosomes (Bean et al., 2000; Bache et al., 2003) but its role in this complex remains uncharacterized. To explore the possibility of an interaction between the Eps15b isoform and Hrs, we performed an immunoprecipitation with anti-Hrs in HEp-2 cells. Unexpectedly, we could not detect any Eps15 in the resulting immunoprecipitate, whereas the Eps15b isoform clearly coimmunoprecipitated with Hrs (Fig. 4 A). We additionally established that the related protein Eps15R did not coimmunoprecipitate with Hrs (Fig. 4 B). These results suggest that Eps15b exists in complex with Hrs in HEp-2 cells. The presence of Eps15b rather than Eps15 in complex with Hrs was a surprising finding, and we therefore wanted to verify the existence of this complex using a different approach. To this end, we depleted HEp-2 cells of Hrs by siRNA. By comparing the levels of Eps15 and Eps15b in Hrs-depleted and control cells, we detected lower levels of Eps15b in Hrs-depleted cells, whereas Eps15 remained unaltered (Fig. 4 C). This result indicates that when cells are depleted of Hrs, Eps15b becomes destabilized, a phenomenon often observed with proteins coexisting in tight complexes.

To further investigate the differential behavior of Eps15 and Eps15b, we compared the distribution of the two proteins in size exclusion chromatography, whereby protein complexes are

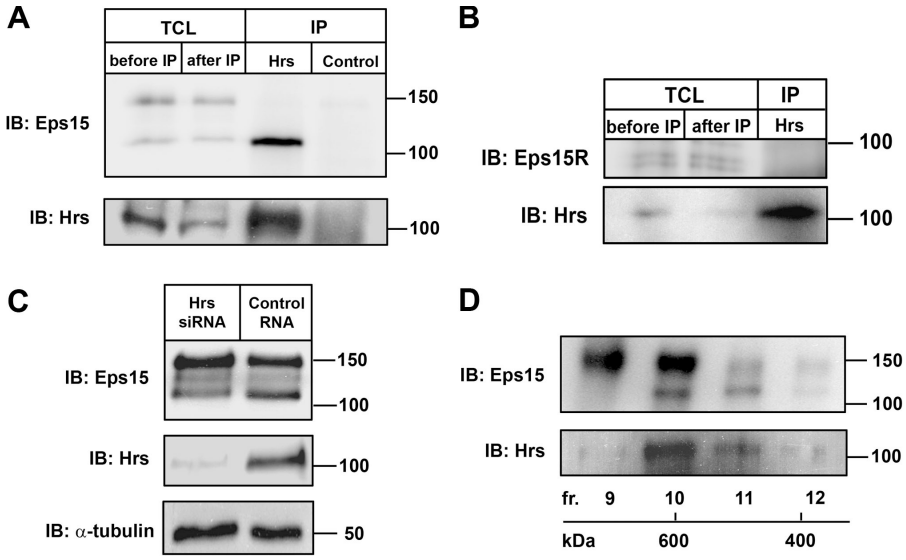


Figure 4. **Eps15b forms a complex with Hrs in vivo.** (A) HEp-2 cells were lysed and immunoprecipitated with anti-Hrs as described in Materials and methods. The immunoprecipitate was analyzed by SDS-PAGE and immunoblotting with anti-Eps15 and anti-Hrs. (B) An immunoprecipitation experiment was performed with anti-Hrs as in A and analyzed using anti-Eps15R and anti-Hrs. (C) HeLa cells were depleted of Hrs by siRNA-mediated knockdown and analyzed by SDS-PAGE and immunoblotting with anti-Eps15, anti-Hrs, and anti-tubulin as a loading control. (D) A431 cells were subjected to size exclusion chromatography separating protein complexes after their molecular weight, and the resulting fractions were immunoblotted with anti-Eps15 and anti-Hrs. Numbers to the right of gel blots indicate molecular mass in kD.

separated on the basis of their molecular weight. Size exclusion chromatography from A431 total cell lysates revealed a differential distribution of Eps15 and Eps15b, with Eps15 being detected in fractions containing complexes of higher molecular weight than Eps15b (Fig. 4 D). In comparison to this, Hrs was detected with the same distribution as Eps15b. Even without precisely determining the sizes of the different complexes, this result indicates that Eps15 and Eps15b form separate protein complexes in vivo and further pinpoints Eps15b to the Hrs complex.

Through the above approaches, we have obtained results suggesting that Eps15b rather than Eps15 exists in complex with Hrs. However, it has been established that the Eps15 protein can interact with Hrs (Bean et al., 2000; Bache et al., 2003) and that both Eps15 and Eps15b contain the domains believed to be responsible for the direct interaction with Hrs. To further characterize the interaction between Hrs and Eps15/Eps15b, we performed in vitro pull-down assays using a GST-Hrs(1–500) fusion protein and in vitro translated Eps15 or Eps15b. As shown in Fig. 5 A, both Eps15 and Eps15b can directly interact with Hrs in vitro, and a comparison of the amount of Eps15 and Eps15b pulled down with GST-Hrs(1–500) revealed that approximately equal amounts of Eps15 and Eps15b were pulled down. To be able to explain the preference of Hrs to interact with Eps15b in vivo by differences in binding affinity, we would expect a large difference in binding between Eps15 and Eps15b in the GST pull-down assays. As we did not observe such a difference, we conclude that the apparent preference of Hrs to bind Eps15b in vivo must depend upon other factors. The endocytic adaptor AP2 is known to exist in complex with the major fraction of Eps15 both in the cytosol and at the plasma membrane (Benmerah et al., 1995; Tebar et al., 1996), and this could potentially render only Eps15b accessible for Hrs and explain the specific formation of an Hrs–Eps15b complex. To investigate whether AP2 preferentially binds full-length Eps15 in vivo, we performed immunoprecipitation experiments with anti–α-adaptin in HEp-2 cells (Fig. 5 B). The resulting immunoprecipitate contained large amounts of Eps15 and only trace amounts of the Eps15b isoform. In summary, our results indicate that although Eps15 and Eps15b appear to have similar capacities to interact with Hrs in vitro, endogenous AP2 interacts with Eps15, whereas Hrs forms a tight complex with the Eps15b isoform.

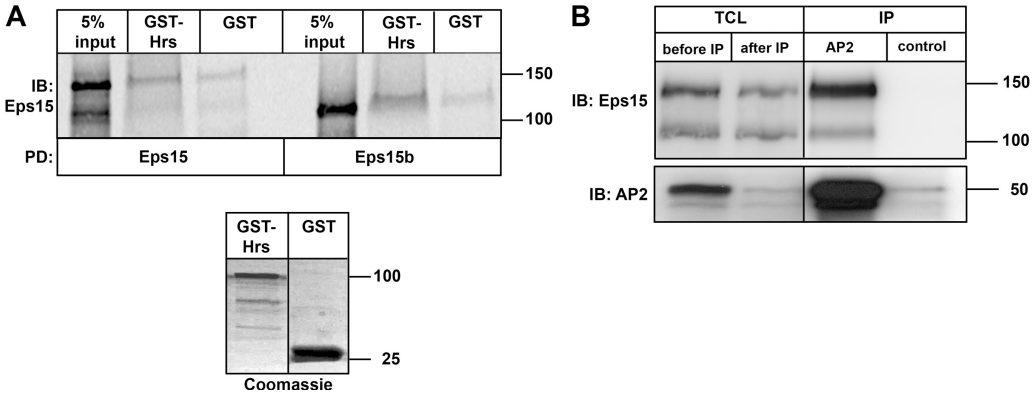


Figure 5. **Both Eps15 and Eps15b interact with Hrs in vitro, whereas only Eps15 shows strong interaction with AP2 in vivo.** (A) In vitro translated $^{35}$S-labeled Eps15 and Eps15b were pulled down with recombinant GST-Hrs(1–500) or GST immobilized on glutathione-Sepharose beads, and the beads were analyzed by SDS-PAGE and autoradiography. (B) Hep-2 cells were lysed and immunoprecipitated with anti-adaptin as described in Materials and methods. The immunoprecipitate was analyzed by SDS-PAGE and immunoblotting using anti-Eps15 and anti-adaptin.

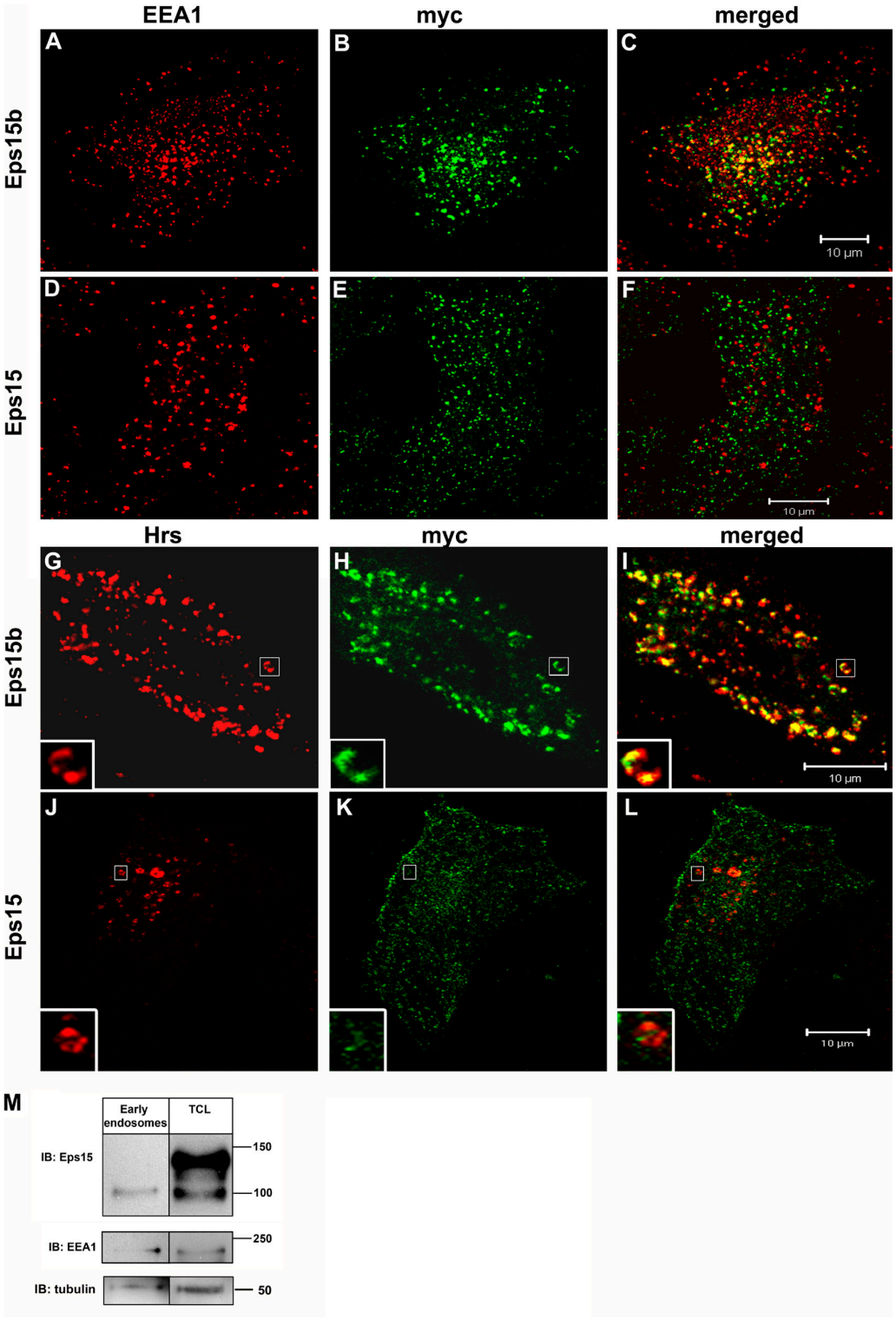


Figure 6. **Eps15b is localized to Hrs-positive microdomains on early endosomes.** HeLa cells grown on coverslips were transiently transfected with myc-Eps15b (A–C) or myc-Eps15 (D–F) for 20 h, permeabilized before fixation, and double labeled with anti-myc (green) and anti-EEA1 (red). Colocalization is indicated in yellow. HeLa cells grown on coverslips were transiently transfected with the constitutively active Rab5 mutant $Rab5^{Q79L}$ for 24 h, generating enlarged endosomes, followed by a transfection with myc-Eps15b (G–I) or myc-Eps15 (J–L) for an additional 24 hr. The cells were permeabilized before fixation and double labeled with anti-Hrs (red) and anti-myc (green). Colocalization is indicated in yellow. Insets show examples of endosomes that are sufficiently large to enable a distinction between different membrane domains. (M) HEp-2 cells were fractionated as described previously (Felberbaum-Corti et al., 2005) and the resulting early endosome–enriched fraction was analyzed by SDS-PAGE and immunoblotting with anti-Eps15, anti-EEA1, and anti–α-tubulin as loading controls. Bars, 10 μm.

### Eps15b is localized to early endosomes

Eps15 localizes to the cytosol and to clathrin-coated pits at the plasma membrane. Additionally, confocal immunofluorescence analysis has identified a small pool of an Eps15 immunoreactive protein present on early endosomes, colocalizing with Hrs and clathrin (Bache et al., 2003). However, the polyclonal antibody used in this analysis cannot distinguish between Eps15 and Eps15b, and we therefore wanted to investigate the specific intracellular localization of Eps15b. Because our attempt to generate an Eps15b-specific antibody failed (unpublished data), we transiently transfected HeLa cells with myc epitope–tagged Eps15b. As shown in Fig. 6 (A–C), Eps15b colocalized with the early endosomal antigen 1 (EEA1) and did not display any plasma membrane staining. This indicates that Eps15b is specifically localized to early endosomes. In cells expressing myc-tagged Eps15, we observed Eps15 in punctuate structures at the plasma membrane and little colocalization with EEA1 (Fig. 6, D–F). However, in cells expressing large amounts of myc-Eps15, a substantial fraction of the protein became localized to endosomal structures (unpublished data), indicating that there is a finely regulated balance between Eps15 and Eps15b regarding their intracellular localization. By transfecting cells with the constitutively active mutant of the small GTPase Rab5, $Rab5^{Q79L}$, we generated enlarged endosomes, where distinct endosomal microdomains can be observed by confocal immunofluorescence microscopy. Myc-tagged Eps15b was found to localize to Hrs-positive microdomains of these enlarged endosomes (Fig. 6, G–I), which is in line with the hypothesis that Eps15b and Hrs coexist in a complex, whereas Eps15 showed punctuate plasma membrane localization (Fig. 6, J–L). To obtain more physiologically relevant information about the distribution of Eps15 and Eps15b, we purified fractions enriched in early endosomes from HEp-2 cells (Felberbaum-Corti et al., 2005). Western blot analysis revealed the resulting fractions to be highly enriched with Eps15b relative to Eps15 (Fig. 6 M). This supports the confocal immunofluorescence analysis and indicates that Eps15b is localized to early endosomes.

### Eps15b mediates endosomal sorting of the EGFR

The Hrs complex constitutes an essential sorting step for ubiquitinated cargo destined for lysosomal degradation, and both overexpression and depletion of Hrs lead to impaired degradation of internalized EGFR (Raiborg et al., 2001a; Bache et al., 2003; Petiot et al., 2003). To address the possible function of Eps15b in endosomal protein sorting, we first asked whether overexpression of Eps15 or Eps15b affects degradation of internalized EGFRs. Interestingly, when we overexpressed myc epitope–tagged Eps15b, degradation of activated EGFR was significantly inhibited (Fig. 7 A), whereas endocytosis of the receptor remained unaltered (Fig. 7 B) as measured by confocal immunoflourescence microscopy. Furthermore, the EGFR accumulated on early endosomes in the myc-Eps15b–expressing cells (Fig. 7, C–F) similarly to Hrs overexpression. In contrast, overexpressing myc-Eps15 did not alter EGFR degradation (Fig. 7, A and G–J). These results show that overexpression of Eps15b causes similar inhibitory effects on EGFR degradation as overexpression of Hrs, which is consistent with the possibility that Eps15b plays a role in EGFR sorting.

To obtain a more direct evidence for a functional role of Eps15b in the Hrs complex, we transfected cells with an siRNA oligonucleotide targeting Eps15 and Eps15b and measured the amount of EGFR remaining in the cells at different time points after stimulation with EGF by confocal immunoflourescence microscopy. In the resulting Eps15- and Eps15b-depleted cells, there was only a minor decrease in endocytosed EGFRs (Fig. 8 A), which was in line with previous studies (Huang et al., 2004; Sigismund et al., 2005). This indicates that neither Eps15 nor Eps15b are essential for EGFR internalization. Interestingly, however, when we assayed degradation of EGFRs, we observed a strongly delayed degradation in Eps15/Eps15b-depleted cells (Fig. 8 B). This indicates that Eps15 and/or Eps15b is involved in ligand-induced degradation of the EGFR independently of the internalization step.

To verify these results biochemically and determine the relative importance of Eps15 and Eps15b, we studied the rates of internalization and degradation of $^{125}$I-labeled EGF in control cells versus Eps15/Eps15b-depleted cells or cells depleted of only Eps15. Consistent with previous studies and with our confocal microscopy analysis, the rate of [$^{125}$I]EGF internalization was slightly reduced in cells depleted of Eps15, and the same was the case in cells depleted of both Eps15 and Eps15b (Fig. 8 C). More importantly, although depletion of Eps15 had no effect on degradation of endocytosed [$^{125}$I]EGF, the additional depletion of Eps15b markedly inhibited [$^{125}$I]EGF degradation (Fig. 8 D). This indicates that Eps15b is required for efficient degradation of endocytosed EGF.

Because depletion of Hrs causes increased recycling of [$^{125}$I]EGF (Raiborg et al., 2008), we next asked if the same is the case when Eps15b is knocked down. Interestingly, our biochemical analysis showed that recycling of [$^{125}$I]EGF was strongly increased in cells depleted of both Eps15 and Eps15b, whereas cells only depleted of Eps15 showed the same rates of [$^{125}$I]EGF recycling as cells treated with a control RNA duplex (Fig. 8 E). This indicates that depletion of Eps15b, similarly to that of Hrs, shifts the trafficking of internalized EGF from the degradative to the recycling pathway. Collectively, our results are consistent with the possibility that Eps15b functions in a complex that recognizes ubiquitinated receptors in the endosomal membrane, thereby promoting their degradation and preventing their recycling.

### Reintroduction of siRNA-resistant Eps15b rescues the impaired EGFR degradation in Eps15/Eps15b-depleted cells

Because siRNA oligonucleotides may sometimes cause off-target effects, we found it important to verify that the inhibitory effect of the siRNA against Eps15/Eps15b on EGFR degradation was specifically caused by knockdown of Eps15b. For this purpose we transfected cells depleted of Eps15 and Eps15b with siRNA-resistant myc-Eps15b or myc-Eps15 and assayed degradation of the EGFR by confocal immunofluorescence microscopy. Knowing that high-level overexpression of myc-Eps15b inhibits EGFR degradation by itself (Fig. 7 A), we studied cells

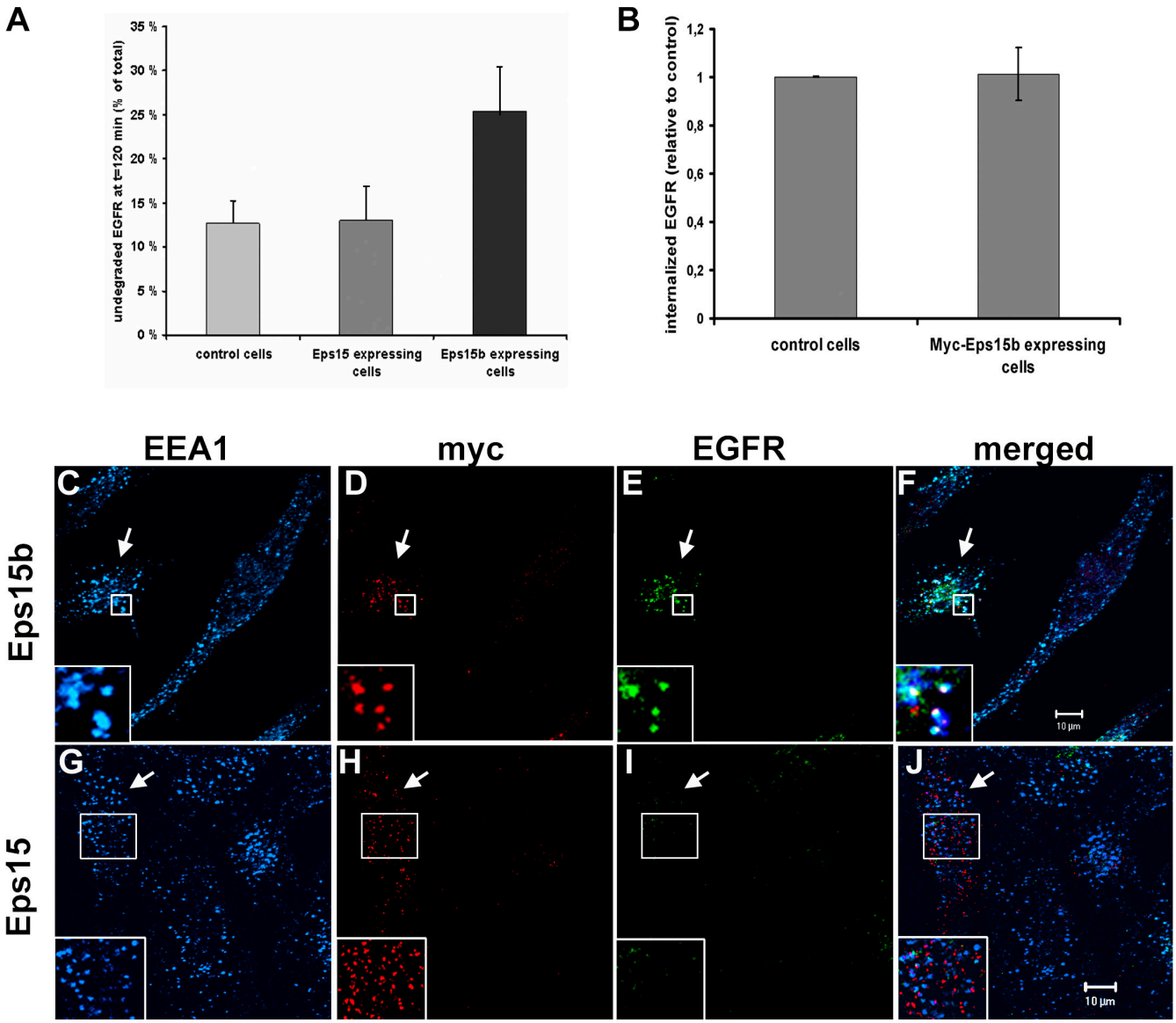


Figure 7. **Overexpression of myc-Eps15b leads to an impairment of EGFR down-regulation independently of endocytosis.** (A) HeLa cells grown on coverslips were transfected with myc-Eps15b or myc-Eps15 for 24 h and subjected to EGF stimulation for 15 or 120 min. The cells were permeabilized, fixed, and stained with anti-EGFR. EGFR degradation was analyzed by quantitating the amount of undegraded EGFR remaining in the cells at t = 120 min relative to t = 15 min by confocal immunofluorescence microscopy. Error bars represent standard errors (control cells, *n* = 18; Eps15-expressing cells, *n* = 10; and Eps15b-expressing cells, *n* = 11). (B) HeLa cells grown on coverslips were transfected with myc-Eps15b for 24 h and stimulated with EGF for 5 min. The EGFR remaining on the cell surface was removed by a 5-min incubation with stripping buffer followed by permeabilization, fixation, and staining with anti-EGFR. Internalized EGFR was quantitated by confocal immunofluorescence microscopy and transfected cells were detected with anti-myc. The figure shows the mean of three independent experiments and error bars represent standard deviations. (C–F) myc-Eps15b– or (G–J) myc-Eps15–expressing cells were subjected to the EGFR degradation assay as in A and triple-stained with anti-EEA1 (blue), anti-myc (red), and anti-EGFR (green). Triple colocalization is indicated in white. Arrows indicate transfected cells. Insets show magnified examples of colocalization.

expressing low amounts of myc-Eps15b. Importantly, although reexpression of myc-Eps15 in siRNA-treated cells did not rescue the inhibited EGFR degradation, reintroducing low levels of Eps15b to these cells did (Fig. 9, A–C). These results indicate that it is indeed the Eps15b isoform that is required for proper down-regulation of the EGFR.

## Discussion

In this work, we have identified a novel Eps15 isoform that is conserved in birds and mammals and named it Eps15b. Although we have been unable to identify direct Eps15b counterparts in invertebrates and lower eukaryotes, it is worth noting that such organisms often contain several Eps15-like molecules that might potentially play differential roles in endocytic trafficking. Although Eps15 has previously been suggested to localize to the Hrs complex on early endosomes, our results indicate that the Eps15b isoform is the main Eps15-related protein species present in this complex, and in contrast to full-length Eps15, it does not localize to clathrin-coated pits and interacts with AP2 only to a minor extent. Our results further indicate that Eps15b, in contrast to Eps15, is required for efficient degradative

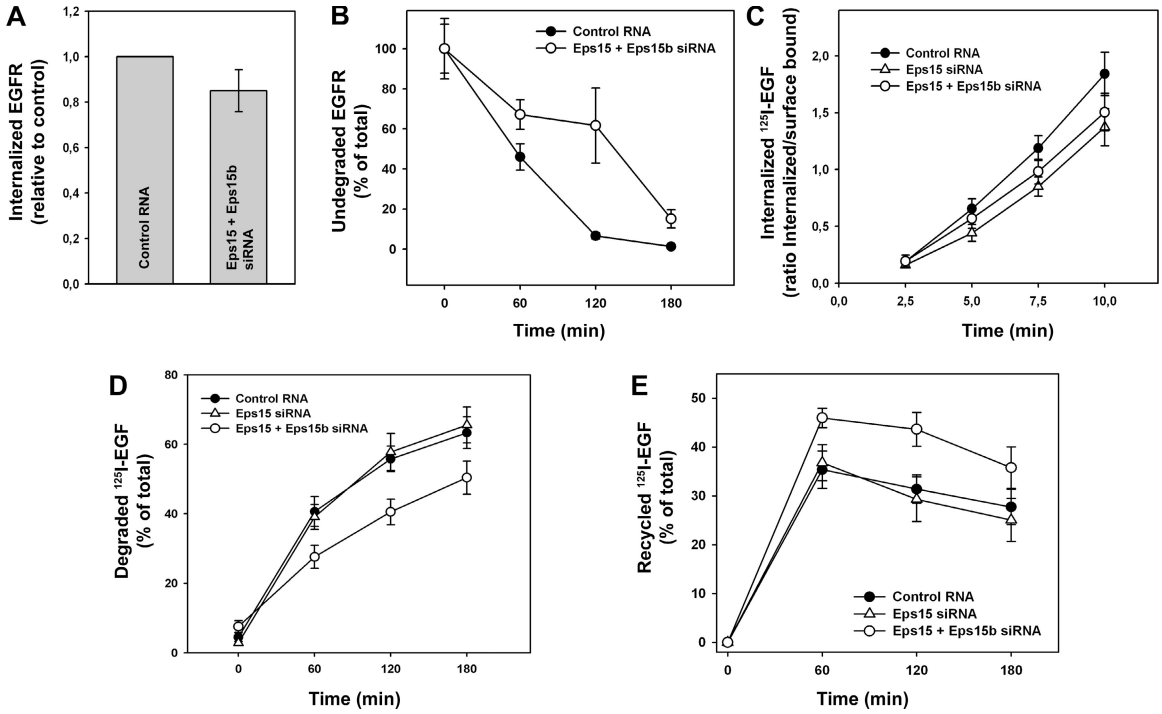


Figure 8. **Eps15b mediates down-regulation of the EGFR independently of endocytosis.** (A) HeLa cells grown on coverslips were depleted of Eps15 and Eps15b by siRNA-mediated knockdown and stimulated with EGF for 5 min at 37°C to induce EGFR internalization. The EGFR remaining on the cell surface was removed by stripping buffer followed by permeabilization, fixation, and staining with anti-EGFR. Internalized EGFR was quantitated by confocal immunofluorescence microscopy. The figure shows the mean of three independent experiments and the error bars represent SEM. (B) Eps15/Eps15b-depleted HeLa cells grown on coverslips were subjected to EGF stimulation for 15, 60, 120, or 180 min. The cells were stained with anti-EGFR. EGFR degradation was analyzed by quantitating the amount of undegraded EGFR remaining in the Eps15/Eps15b knockdown and in cells treated with a control RNA duplex at each time point by confocal immunofluorescence microscopy. Error bars represent SEM (control: 15 min, $n = 22$; 60 min, $n = 22$; 120 min, $n = 22$; 180 min, $n = 27$; and Eps15+Eps15b siRNA: 15 min, $n = 23$; 60 min, $n = 24$; 120 min, $n = 23$; and 180 min, $n = 21$). (C) Internalization of [$^{125}$I]EGF in HeLa cells depleted of Eps15/Eps15b. Eps15/Eps15b-depleted cells were seeded in 24-well plates and an internalization assay of [$^{125}$I]EGF (1 ng/ml) was preformed as described in Materials and methods. Internalized [$^{125}$I]EGF was calculated as counts per minute of internalized [$^{125}$I]EGF divided by counts per minute of surface-localized [$^{125}$I]EGF. The data represent six independent experiments with four parallels. The difference between control and both Eps15- and Eps15/Eps15b-depleted cells was statistically significant at time points 5, 7.5, and 10 min ($P < 0.05$). Error bars represent SEM. (D and E) Degradation (D) and recycling (E) of [$^{125}$I]EGF in Eps15/Eps15b-depleted cells. Cells were incubated with [$^{125}$I]EGF (1 ng/ml) on ice for 15 min then washed with PBS before preheated medium was added to the cells and chased for the indicated time period at 37°C. Upon incubation, the medium was divided into acid-soluble and -precipitable fractions representing degraded and recycled [$^{125}$I]EGF, respectively. The cell-associated radioactivity represented internalized and cell-associated [$^{125}$I]EGF. The data are shown as a percentage of total initially bound [$^{125}$I]EGF and represent six independent experiments with four parallels. There was no statistically significant difference between control and Eps15-depleted cells with regard to degradation and recycling, but for Eps15b depleted cells, the difference to control cells was statistically significant ($P < 0.05$) for all time points (60, 120, and 180 min). Error bars represent SEM.

endosomal sorting of EGFRs. We propose that Eps15 and Eps15b function in complexes that mediate cargo recognition at the plasma membrane and the endosome, respectively (Fig. 10).

The Eps15b isoform has not previously been characterized, and by combining sequence information available in public databases and experimental approaches, we demonstrate that Eps15b is evolutionarily conserved and expressed in several human cell types. The Eps15b isoform contains 32 amino acid residues in its N-terminal end, which in the full-length Eps15 are spliced out from the precursor mRNA as a part of intron 12. Various bioinformatic search tools did not identify any known motives within this sequence but these 32 residues could nevertheless give the Eps15b protein different properties compared with full-length Eps15. Even more important for the properties of Eps15b might be the lack of the three N-terminal EH domains, which in full-length Eps15 are essential for its participation in the complex network of multiple protein–protein interactions at the clathrin-coated pits. The capacity of Eps15b to participate in protein–protein interactions is consequently significantly smaller than for Eps15. The Eps15b isoform has the same apparent molecular weight as a putative Eps15 isoform discovered by Fazioli et al. (1993) in rodent NIH 3T3 cells. Although we have not addressed the specific identity of the protein identified by Fazioli et al., it is not unlikely that this protein could correspond to the Eps15b isoform. In their work, they observed this 120-kD protein as a doublet band, similarly to the Eps15 doublet band at 150 kD, which was later shown to correspond to the ubiquitinated and nonubiquitinated form of Eps15. Furthermore, both the 150-kD Eps15 protein and the 120-kD protein were tyrosine phosphorylated upon EGF stimulation. Thus, the Eps15b protein may share the general characteristics of EGF-induced phosphorylation and monoubiquitination with Eps15.

Although an Eps15 reactive species has previously been identified as a component of the Hrs complex on early endosomes, we have gathered data indicating that the Eps15b isoform is the main product of the *EPS15* gene stably associated with Hrs. In vitro binding assays further indicated that the specific formation of an Hrs–Eps15b complex is based upon other factors than differences in interaction affinity. The fact that

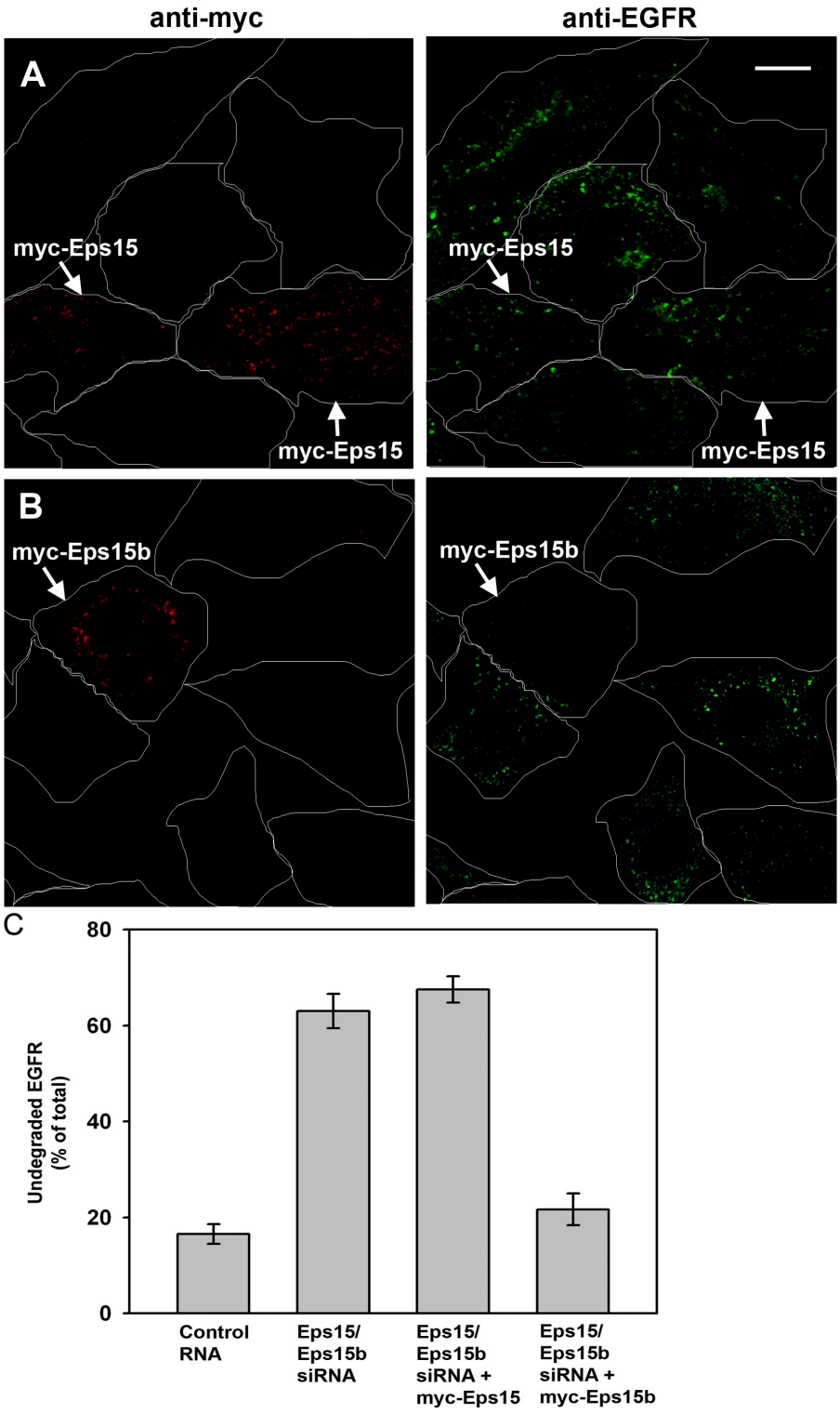


Figure 9. **Eps15b is essential for EGFR degradation.** HeLa cells were treated with Eps15- and Eps15b-specific siRNA oligonucleotides and at the same time transiently transfected with siRNA-resistant myc-Eps15 (A) or myc-Eps15b (B). The cells were stimulated with 50 ng/ml EGF for 15 min and then washed and incubated for 2 h in the presence of cycloheximide to allow degradation of internalized EGFRs. The cells were then prepared for confocal immunofluorescence microscopy and labeled with anti-EGFR (green) and anti-myc (red) antibodies. (A) Eps15- and Eps15b-depleted cells transfected with myc-Eps15 (arrows) showed inhibited EGFR degradation similar to Eps15/Eps15b-depleted neighboring cells. (B) Eps15- and Eps15b-depleted cells transfected with myc-Eps15b (arrows) showed normal degradation of EGFR as compared with Eps15/Eps15b-depleted neighboring cells in which the degradation was inhibited. White lines indicate the cell borders. Bar, 10 µm. (C) The amount of EGFR remaining after 2 h of chase was quantified as described in Materials and methods and is represented as percentage of the total amount of internalized EGFR after 15 min. Control cells were treated with a nontargeting RNA duplex. In total, 100–300 cells were measured for each treatment. Error bars show ± SEM from four independent experiments.

Eps15 constitutively interacts with AP2 may provide a possible explanation, as Hrs and AP2 are not able to bind Eps15 simultaneously (Bean et al., 2000). Immunoprecipitation experiments established that AP2 mainly interacts with Eps15 in vivo, and only small amounts of Eps15b were coimmunoprecipitated with AP2. Consequently, with Eps15 constitutively bound to AP2, only Eps15b will be accessible to form a complex with Hrs. Given the fact that Eps15b and Eps15 both contain the DPF repeats capable of interacting with AP2, these results raise the question of why AP2 does not bind Eps15b in vivo. One possible explanation could be provided by differences in oligomerization status between the Eps15 and Eps15b isoforms. Eps15 exists as dimers and tetramers in cells, although it is unclear whether dimer or tetramer is the predominant form (Tebar et al., 1997). The formation of antiparallel tetramers depends on the N- and C-terminal domains of the protein (Cupers et al., 1997). As Eps15b

lacks the N-terminal domain, it is not able to form tetramers but may still form dimers. This could possibly explain the reduced AP2 binding because AP2 has been shown by immunoprecipitation experiments to bind tetramers of Eps15 significantly more efficiently than dimers (Tebar et al., 1997). A second possible explanation is that the interaction between AP2 and Eps15 is mediated or enhanced by an unknown protein, potentially through the EH domains that would exclude the Eps15b isoform from the interaction.

Our combined biochemical and immunofluorescence-based approaches indicate that the Eps15b isoform is localized to early endosomes and not to clathrin-coated pits. It has previously been shown that an Eps15 deletion mutant lacking the second and third EH domains loses correct targeting to clathrin-coated pits and instead is cytosolic (Benmerah et al., 1999). It is therefore not surprising that Eps15b, which lacks all three EH domains, is not localized to clathrin-coated pits, although its localization to endosomes was unanticipated. Eps15 mainly localized to clathrin-coated pits in our assays, but Eps15 may also be localized to endosomal compartments during EGF stimulation of cells. It has been suggested that Eps15 may be internalized together with the clathrin-coated vesicles and further transported to early endosomes together with the EGFR, or alternatively, dissociates from the forming clathrin-coated vesicle followed by a retargeting to endosomes (Tebar et al., 1996; van Delft et al., 1997; Cupers et al., 1998; Torrisi et al., 1999). Although controversial, these remain putative mechanisms for Eps15 localization to endosomal compartments. However, some of these studies have been performed with antibodies that cannot distinguish between Eps15 and Eps15b and it is therefore unclear which isoform contributes to the distinct parts of the distribution patterns. Our studies indicate that Eps15b is the predominant Eps15 isoform on endosome membranes and that the immunolabeling within bilayered coats on endosomes (Fig. 1) most likely represents Eps15b.

To address the functional role of Eps15b in the Hrs complex, we used both overexpression and siRNA-based approaches. Overexpression of Eps15b led to impaired EGFR degradation. This phenotype is identical to what is observed in cells overexpressing Hrs and suggests that the Eps15b isoform plays a role in endosomal protein sorting as a part of the Hrs complex. The effect could be caused by the accumulation of Hrs on early endosomes (unpublished data). Importantly, overexpression of Eps15 did not lead to any inhibition of EGFR degradation, which suggests that the role in degradative EGFR sorting is specific to Eps15b. Because the mechanism by which overexpression of Eps15b or Hrs prevents EGFR degradation is not clear, we also sought to measure degradation of the EGFR in cells depleted of Eps15b. Because we were unsuccessful in generating an siRNA oligonucleotide that specifically knocked down Eps15b, we performed the assay using an oligonucleotide targeting both Eps15 and Eps15b. In cells depleted of Eps15 and Eps15b but not in cells depleted of only Eps15, the degradation of the EGFR was impaired. Importantly, this inhibitory phenotype was rescued by the reintroduction of low levels of siRNA-resistant Eps15b but not Eps15. This indicates that Eps15b is required for degradation of endocytosed EGFRs.

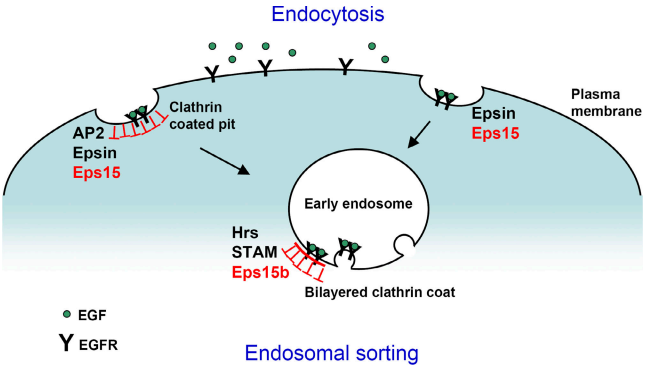


Figure 10. **Differential functions of Eps15 and Eps15b in EGFR trafficking.** Together with AP2, Epsin, and several other proteins, Eps15 mediates internalization of activated EGFRs. The endocytosed receptors and their ligands are then transported to early endosomes, where they are retained in the bilayered clathrin coat by Hrs, STAM, and Eps15b. They are further escorted into intraluminal vesicles of multivesicular endosomes by ESCRT I–III and finally degraded in lysosomes (not depicted).

Which mechanism could account for the inhibited degradation of EGFRs observed in Eps15b-depleted cells? Previous studies have shown that depletion of Hrs or ESCRT-I enhances recycling of endocytosed EGF and its receptor, whereas this is not the case with depletion of ESCRT-II and ESCRT-III (Bache et al., 2006; Raiborg et al., 2008). Following the fate of internalized [$^{125}$I]EGF, we observed that recycling of this ligand was strongly enhanced in cells treated with siRNA against Eps15 + Eps15b but not in cells only depleted of Eps15. Thus, depletion of Eps15b results in the same net effect as depletion of Hrs, which is consistent with the idea that these proteins function in the same endosomal sorting complex. We speculate that the role of this complex may be to recognize ubiquitinated cargoes such as activated EGFRs in the endosomal membrane, thereby preventing their recycling to the plasma membrane.

Several alternative mechanisms have been suggested for the role of Eps15 in endocytosis. One hypothesis suggests that Eps15 recruits ubiquitinated receptors to the rims of clathrin-coated pits by interacting with the ubiquitin tag through its UIMs (Polo et al., 2002; Riezman, 2002; de Melker et al., 2004; Stang et al., 2004). Eps15 has also been found to be involved in clathrin-dependent endocytosis of nonubiquitinated EGFRs as well as clathrin-independent endocytosis of ubiquitinated EGFRs (Sigismund et al., 2005), which suggests that the function of Eps15 extends beyond clathrin- and ubiquitin-dependent receptor trafficking. Although ubiquitination of membrane proteins is a dominant signal for their endosomal sorting, only a few proteins have been shown to use ubiquitin as an endocytosis signal (Hicke and Dunn, 2003; Raiborg et al., 2003). The function of the Eps15b isoform in the Hrs complex could be to increase the avidity of the complex for ubiquitinated cargo. Recently, it was shown in *Drosophila melanogaster* that Eps15 is required for synaptic vesicle endocytosis and that the loss of Eps15 reduces the amount of dynamin and Dap160/Intersectin at the neuromuscular junctions, which indicates a role of Eps15 in maintaining dynamin at the site of endocytosis (Koh et al., 2007). Eps15 has additionally been shown to stimulate clathrin

assembly at clathrin-coated pits (Morgan et al., 2003). The Hrs-positive microdomains on early endosomes contain clathrin coats, and to address whether the Eps15b isoform stimulates clathrin assembly in these microdomains, we investigated the localization of clathrin on endosomes in cells depleted of Eps15 and Eps15b and in cells expressing large amounts of myc-Eps15b. We could not detect any significant effects (unpublished data), which suggests that Eps15 proteins are not essential for clathrin assembly on early endosomes.

In conclusion, we have identified Eps15b as an evolutionarily conserved and functionally distinct isoform of Eps15. Our results suggest that Eps15b, in contrast to Eps15, constitutes a stable part of the Hrs complex and mediates endosomal sorting of EGFRs (Fig. 10). In future experiments, it will be interesting to elucidate the exact functions of Hrs, STAM, and Eps15b in this endosomal sorting complex.

## Materials and methods

### Plasmid constructs and siRNA

The Eps15b full-length cDNA clone DKFZp686D16141 was obtained from imaGenes and the myc-Eps15b construct was generated by PCR amplification and cloning into pcDNA3-myc. The myc-Eps15 construct was generated by subcloning the cDNA into pcDNA3-myc. The pcDNA3-Rab5$^{Q79L}$ has been described previously (Stenmark et al., 1994). A GST-Hrs (residues 1–500 of Hrs) construct was generated by subcloning the cDNA into pGEX-6P (GE Healthcare). The siRNA oligos against the C terminus of Eps15 and Eps15b and the N terminus of Eps15 have been described previously (Huang et al., 2004; Sigismund et al., 2005). The sense sequence of the Eps15-specific RNAi was GAGUUUGGGAGUUGAGUGA and the sense sequence of the Eps15/Eps15b specific RNAi was AAACGGAGCUACAGAUUAU. A nontargeting siRNA oligo obtained from Dharmacon was used as a negative control. A PCR primer was designed to generate an siRNA-resistant Eps15b with the sequence GCATTCTTGTAAATGGCGCCACTGATTATTGCAGCCTC. The siRNA specific for Hrs has been described previously (Bache et al., 2003).

### Confocal immunofluorescence microscopy

HeLa cells grown on coverslips were permeabilized with 0.05% saponin, fixed with 3% paraformaldehyde, and stained for fluorescence microscopy as described previously (Simonsen et al., 1998). Coverslips were analyzed at 25°C with a confocal microscope (LSM 510 META; Carl Zeiss, Inc.) equipped with Plan Apochromat 63× 1.4 NA and Neo Fluar 100× 1.45 NA oil immersion objectives (both from Carl Zeiss, Inc.). Appropriate emission filter settings were included to exclude bleed-through effects. Images were acquired with the LSM 510 software (version 3.2; Carl Zeiss Inc.) and processed with Photoshop (version 7.0; Adobe).

### Immunoelectron microscopy

HEp-2 and HeLa cells were serum-starved (0.5% FCS) 20 h before incubation with EGF. Precooled cells (left on ice) were incubated with EGF (60 ng/ml in MEM without bicarbonate and with 0.1% BSA) on ice for 15 min followed by washing three times in ice-cold PBS to remove unbound ligand. Subsequently, the cells were chased in ligand-free MEM without bicarbonate at 37°C for 15 min before fixation and preparation for cryosectioning and immunolabeling as described previously (Myromslien et al., 2006). For single labeling, thawed cryosections were labeled using rabbit anti-Eps15 (C terminus) from Covance Research Products followed by 15-nm protein A–gold (obtained from G. Posthuma, Utrecht University, Utrecht, Netherlands). In double-labeling experiments, labeling for Eps15 was followed by incubation with free protein A before labeling for Hrs followed by 10 nm protein A–gold. Sections were examined using a transmission electron microscope (CM120; Philips) with a transmission electron microscopy soft imaging system (Megaview II; Olympus).

### Cell culture and transfection

HeLa and HEp-2 cell cultures were maintained as described by the American Type Culture Collection. pcDNA3 constructs were transfected into cells using FuGENE 6 (Roche) or Effectene (QIAGEN) according to the manufacturer's instructions. Transfection of HeLa cells with siRNA oligonucleotides (200 nM) was performed as described previously (Elbashir et al., 2002) using Oligofectamine (Invitrogen).

### Antibodies

Rabbit anti-Eps15 (raised against a peptide in the C terminus; C-20) and mouse anti–α-adaptin (C8) were obtained from Santa Cruz Biotechnology, Inc. Two additional Eps15 antibodies were used, a rabbit anti-Eps15 obtained from Abcam (raised against amino acids 539–896) and a rabbit anti-Eps15 (C terminus; 569–896) obtained from Covance. The N-terminal mouse anti-Eps15 antibody 6G4 (Benmerah et al., 1995) was a gift from A. Benmerah (Institut Cochin, Paris, France). The rabbit anti-Eps15R antibody was a gift from S. Polo (Instituto Firc di Oncologia Molecolare, Milan, Italy). Sheep anti-EGFR was obtained from Fitzgerald. Rabbit antibodies against recombinant Hrs have been described previously (Raiborg et al., 2001a). Mouse anti-myc antibodies were derived from 9E10 hybridoma cells. Human anti-EEA1 antiserum (Mu et al., 1995) was a gift from B.-H. Toh (Monash University, Melbourne, Australia). The mouse monoclonal antibody against α-tubulin was obtained from Sigma-Aldrich. Cy2-, Cy3-, and horseradish peroxidase–labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

### Immunoprecipitation experiments

HEp-2 cells were lysed (lysis buffer: 25 mM Hepes, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 0.5% Igepal CA-630, 1 mM dithiothretiol, and mammalian protease inhibitor mixture) and precleared by incubating at 4°C for 1 h with 50 µl protein A–Sepharose (GE Healthcare) preincubated with rabbit or mouse IgG followed by an incubation at 4°C for 1 h with 50 µl protein A–Sepharose preincubated with 5 µl anti-Hrs serum or 3 µg anti–α-adaptin. Immunoprecipitates were washed and analyzed by SDS-PAGE (precast gels were obtained from Thermo Fisher Scientific) and immunoblotting.

### Real-time PCR

Total RNA was isolated from HeLa cells 3 d after siRNA transfection using the Aurum Total RNA mini kit (Bio-Rad Laboratories) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) as described by the manufacturer. Real-time PCR was performed with manually designed Ep15- and Eps15b-specific primers (Eps15b forward primer, 5′-CTTGCATTGTTTGCTGGT-3′; Eps15b reverse primer, 5′-CTTGAAGATCCTBGAACCTC-3′; Eps15 forward primer, 5′-CCTGTTGCAGATTTCTCTG-3′; and Eps15 reverse primer, 5′-TCATCTTGAAGATCCTGAAC-3′) and tyrosyl-tRNA synthetase (YARS) as an internal reference control (YARS forward primer, 5′-GCCTACCCAGATCCCTCAAAG-3′; YARS reverse primer, 5′-ATGACCTCCTCTGGTTCTGAATTC-3′). One primer out of each primer set was designed spanning an intron-exon boundary to avoid amplification of contaminating DNA. The amplifications were performed with a LightCycler 480 instrument (Roche) according to the manufacturer's instructions. The quantification values of Eps15/Eps15b relative to YARS were calculated using the LightCycler relative quantification software. Standard curves were generated to calculate the efficiency of each primer set and then integrated into the data analysis.

### GST pull-down assays

Eps15 and Eps15b were in vitro transcribed and translated with the TNT T7 Coupled Reticulocyte lysate system (Promega) as described by the manufacturer in the presence of [$^{35}$S]methionine (PerkinElmer). The GST-Hrs fusion protein was expressed by transforming *Escherichia coli* BL-21 (DE3) with the construct, and protein expression was induced by 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C and the concentration was measured by Coommassie staining. GST or GST-Hrs was bound to 25-µl aliquots of glutathione-Sepharose (GE Healthcare) at room temperature for 60 min, the beads were washed with assay buffer (25 mM Hepes, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, and 1 mM dithiothretiol), and equal amounts of in vitro translated Eps15 or Eps15b was added. After rotation at 4°C for 60 min, the beads were washed with assay buffer and analyzed by SDS-PAGE and autoradiography.

### Endosome purification

A fraction enriched in early endosomes was generated from HEp-2 cells as described previously (Felberbaum-Corti et al., 2005).

### Size exclusion chromatography

A431 cells were lysed as described previously and lysates were filtered through a 0.22-µm membrane (Millipore). The lysate was fractionated on

a Sepharose 200 gel filtration column using the Äkta Explorer fast protein liquid chromatography system (GE Healthcare). The resulting fractions were TCA precipitated, resuspended in SDS loading buffer, and analyzed by SDS-PAGE followed by immunoblotting with Eps15 and Hrs antibodies.

**Assay of EGFR degradation and internalization**

HeLa cells were depleted of Eps15 and Eps15b by siRNA-mediated knockdown for 72 h, seeded onto coverslips, and cultured for an additional 48 hr. Alternatively, untreated HeLa cells were transfected with myc-Eps15b or myc-Eps15. EGFR degradation was assayed by incubating the cells for 15, 60, 120, or 180 min after stimulating with 100 ng/ml EGF in the presence of 10 µg/ml cycloheximide, followed by fixation and staining with anti-EGFR. EGFR internalization was assayed by incubating siRNA cells with 100 ng/ml EGF for 5 min followed by a 5-min incubation with stripping buffer (2 M NaCl and 20 mM NaAc, pH 4.0) at 37°C to remove all EGFR remaining at the cell surface, and finally fixation and staining with anti-EGFR. The amount of undegraded or internalized EGFR was quantitated by immunoflourescence microscopy. Coverslips were scanned with a LSM 510 Meta microscope at fixed intensity settings below pixel value saturation, and ~20 cells were quantitated from each sample at each time point. The images were processed with the histogram function in the LSM software. The experiments were independently repeated with similar results.

**Assay of rescuing EGFR degradation with reexpressed Eps15b**

HeLa cells were first depleted of endogenous human Eps15 and Eps15b by siRNA for 72 h. The siRNA-treated cells were then seeded onto coverslips in a 24-well tissue culture dish and transfected the following day with siRNA-resistant pcDNA3-myc-Eps15b or pcDNA3-myc-Eps15 for 17 h. The transfected cells were preincubated with 20 µg/ml cycloheximide for 1 h before incubation with 50 ng/ml EGF for 15 min in the presence of 20 µg/ml cycloheximide. The cells were washed in PBS and incubated for 2 h in DME supplemented with 10% FCS and 20 µg/ml cycloheximide to allow EGFR degradation. The cells were then processed for confocal microscopy using anti-myc and anti-EGFR antibodies. For the quantification of cell-associated EGFR, confocal images of cells were recorded at fixed intensity settings below pixel value saturation and analyzed by post-image processing. All pixel values above background level (defined as mean values obtained in unlabeled cells) were integrated for each cell using the histogram function in the LSM software. The experiment was repeated four times and in total, 100–300 cells were analyzed from each category. Because high-level overexpression of Eps15b inhibits EGFR trafficking (this paper), only cells expressing low levels of myc-tagged Eps15b constructs (defined as below a threshold value corresponding to close to endogenous levels using the palette function of the LSM software) were included in the analysis.

**Internalization of [$^{125}$I]EGF**

Internalization of [$^{125}$I]EGF was measured upon incubation with 1 ng/ml [$^{125}$I]EGF (GE Healthcare) as described previously (Johannessen et al., 2006). The ratio of internalized to surface-bound EGF was plotted as a function of time.

**Assay for degradation and recycling of [$^{125}$I]EGF**

Analysis of degradation and recycling of [$^{125}$I]EGF was preformed essentially as described previously (Sorkin et al., 1988; Skarpen et al., 1998). In brief, 1 ng/ml [$^{125}$I]EGF in Hepes buffer was added to cells on ice for 15 min. Then the cells were washed three times with ice-cold PBS to remove unbound [$^{125}$I]EGF. The cells were further chased in Hepes buffer at 37°C for the indicated time periods. Upon incubation the cells where placed on ice and the medium was transferred to tubes where the medium was precipitated with 50% TCA and 10% phosphotungstic acid (TCA-PTA) for 1 h at 4°C before the TCA-precipitable and the TCA-soluble radioactivity were separated into two fractions representing recycled (TCA-precipitable) and degraded (TCA-soluble) [$^{125}$I]EGF. Both fractions were analyzed in a $\gamma$ counter. The cells remaining in the wells were washed with ice-cold PBS before 1% SDS in PBS was added and the cells were collected and analyzed in a $\gamma$ counter. This fraction represents internalized and cell-associated [$^{125}$I]EGF.

**Statistical analyses**

Differences between control and treatment groups were tested using a Student's *t* test.

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