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RESEARCH ARTICLE

# The non-receptor tyrosine kinase Ack1 regulates the fate of activated EGFR by inducing trafficking to the p62/NBR1 pre-autophagosome

**Sylwia Jones, Debbie L. Cunningham, Joshua Z. Rappoport* and John K. Heath**


## ABSTRACT

Growth factor signalling regulates multiple cellular functions and its misregulation has been linked to the development and progression of cancer. Ack1 (activated Cdc42-associated kinase 1, also known as TNK2) is a non-receptor tyrosine kinase that has been implicated in trafficking and degradation of epidermal growth factor receptor (EGFR), yet its precise functions remain elusive. In this report, we investigate the role of Ack1 in EGFR trafficking and show that Ack1 partially colocalises to Atg16L-positive structures upon stimulation with EGF. These structures are proposed to be the isolation membranes that arise during formation of autophagosomes. In addition, we find that Ack1 colocalises and interacts with sequestosome 1 (p62/SQSTM1), a receptor for selective autophagy, through a ubiquitin-associated domain, and this interaction decreases upon treatment with EGF, thus suggesting that Ack1 moves away from p62/SQSTM1 compartments. Furthermore, Ack1 interacts and colocalises with NBR1, another autophagic receptor, and this colocalisation is enhanced in the presence of ectopically expressed p62/SQSTM1. Finally, knockdown of Ack1 results in accelerated localisation of EGFR to lysosomes upon treatment with EGF. Structure–function analyses of a panel of Ack1 deletion mutants revealed key mechanistic aspects of these relationships. The Mig6-homology domain and clathrin-binding domain both contribute to colocalisation with EGFR, whereas the UBA domain is essential for colocalisation with p62/SQSTM1, but not NBR1. Taken together, our studies demonstrate a novel role for Ack1 in diverting activated EGFR into a non-canonical degradative pathway, marked by association with p62/SQSTM1, NBR1 and Atg16L.


**KEY WORDS: Ack1/TNK2, EGFR, p62/SQSTM1, NBR1, Autophagy**

## INTRODUCTION

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of cell surface receptor tyrosine kinases (RTKs) (Citri and Yarden, 2006). Ligand binding results in EGFR dimerisation, transphosphorylation and ubiquitylation, leading to activation of several downstream signalling cascades (Levkowitz et al., 1998; Citri and Yarden, 2006; Schneider and Wolf, 2009). Following activation, EGFR undergoes regulated endocytosis from the cell surface (Rappoport and Simon, 2009; Goh et al., 2010). Internalised EGFR is targeted to early endosomes, where it is sorted through the recycling compartment back to the plasma membrane, or into lysosomes for degradation (Madshus and Stang, 2009). Furthermore, endocytosis of EGFR, its incorporation into multivesicular bodies of late endosomes and its lysosomal degradation are essential for signal attenuation, and uncontrolled EGFR signalling has been found in different types of cancer (Burke et al., 2001; Di Fiore and De Camilli, 2001; Seto et al., 2002). Thus, the mechanisms that regulate EGFR trafficking are significant.

Ack1 (activated Cdc42-associated kinase 1 or TNK2) is a non-receptor tyrosine kinase that has been proposed to regulate EGFR trafficking (Grøvdal et al., 2008), yet the precise mechanistic roles of Ack1 in this context remain elusive. High levels of Ack1 expression result in the inhibition of EGFR degradation (Grøvdal et al., 2008), possibly because of the disruption of endocytic machinery as a consequence of clathrin sequestration (Teo et al., 2001). Additionally, downregulation of Ack1 has also been shown to inhibit EGFR degradation and increase recycling, and Ack1 has been proposed to regulate endosomal sorting of EGFR into the inner vesicles of multivesicular bodies (Grøvdal et al., 2008). Interestingly, increased Cdc42-dependent Ack1 phosphorylation has been observed in cells depleted of dynamin, and in these cells, Ack1 showed enhanced binding of both endocytic and ubiquitylated proteins (Shen et al., 2011).

Apart from ‘classical’ lysosomal degradation, other non-canonical degradative pathways exist in which misfolded proteins, protein aggregates, damaged organelles and bacteria are ubiquitylated and degraded (Kraft et al., 2010). Selective autophagy, which eliminates ubiquitylated protein aggregates and organelles, is one of the major degradative pathways within the cell. Formation of protein aggregates has been suggested to be mediated by autophagy receptors, which recognise ubiquitylated cargo: these include sequestosome 1 (p62/SQSTM1) and neighbour of BRCA1 (NBR1) (Kraft et al., 2010). Although autophagy takes place at the basal levels within the cell, there are various stimuli that have been shown to induce autophagy, in particular withdrawal of growth factors (Wang and Levine, 2010; Mizushima et al., 2011), for example, deprivation of EGF in mammary epithelial cells (Fung et al., 2008).

Recently, we reported that NBR1 functions in degradation of RTK (Mardakheh et al., 2009; Mardakheh et al., 2010). Specifically we showed that association of NBR1 with Spred2, a signalling inhibitor, promotes RTK degradation, whereas NBR1 on its own inhibits degradation. In the present study we investigate the precise roles of Ack1 in EGFR trafficking. We find that Ack1 interacts and colocalises with p62/SQSTM1 (Lamark et al., 2009), and this interaction decreases upon EGF stimulation. The UBA domain strongly regulates the association

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between Ack1 and p62/SQSTM1, but not NBR1. Conversely, EGF stimulation results in localisation of Ack1 to Atg16-positive structures, which are likely to be pre-autophagosomal isolation membranes (Matsushita et al., 2007; Mizushima et al., 2011). Furthermore, silencing of Ack1 leads to enhanced EGFR lysosomal localisation. Thus, our results define a novel role for Ack1 in targeting activated EGFR into a non-canonical degradative pathway through its association with autophagic receptors p62/SQSTM1 and NBR1, which influences the kinetics of EGFR trafficking.

## RESULTS

### Ack1 interacts and colocalises with EGFR, but not with FGFR

A role for Ack1 in EGFR trafficking has been proposed (Shen et al., 2007; Grøvdal et al., 2008), yet the precise functions remain elusive. Consistent with previous reports (Shen et al., 2007), we show that EGFR co-precipitates with Ack1 following EGF stimulation (Fig. 1A). Constitutively active Cdc42 (caCdc42), a known Ack1 interactor (Manser et al., 1993), also co-precipitates with Ack1 (Fig. 1A). In contrast, FGFR1 and FGFR2 do not co-precipitate with Ack1 following treatment with FGF (Fig. 1A and supplementary material Fig. S1B). Similarly, Ack1 colocalises with EGFR in EGF-treated cells (Fig. 1B), as represented by the high Pearson's correlation coefficient (PCC), which decreases upon deliberate misalignment by pixel movement (see the Materials and Methods). In the case of FGFR2, the PCC is low and remains low irrespective of pixel movement, indicating a lack of colocalisation between Ack1 and FGFR2 (Fig. 1B). These results emphasise the differences between EGFR and FGFR trafficking, despite activation of similar downstream signalling pathways. Additionally, truncated Ack1 (tAck1), which lacks the C-terminal portion (supplementary material Fig. S1B), does not colocalise with EGFR after EGF treatment (supplementary material Fig. S1C), indicating that the C-terminal fragment is essential for the Ack1–EGFR association. This is consistent with the studies showing that the Mig6 homology domain within the C-terminus of Ack1 mediates this association (Shen et al., 2007).

In order to study the physiological relevance of this association, we took advantage of the human prostatic adenocarcinoma cell line LNCaP, which has previously been used to study endogenous Ack1 (Mahajan et al., 2005; Liu et al., 2010). As shown in Fig. 1C, colocalisation between endogenous Ack1 and EGFR could be detected in LNCaP cells. To our knowledge this is the first successful report of colocalisation between endogenous Ack1 and EGFR. Therefore, under physiological conditions, Ack1 and EGFR show EGF-dependent colocalisation.

### The Mig6-homology domain and clathrin-binding domain of Ack1 both contribute to the colocalisation with EGFR upon EGF stimulation

It has been proposed that the UBA domain of Ack1 is required for EGFR degradation, whereas the Mig6 domain mediates the interaction with EGFR (Shen et al., 2007). To analyse more precisely which of the Ack1 domains are required for the association with EGFR, we generated a series of C-terminal truncations of Ack1 (Fig. 2A). These include a mutant with deletion of the C-terminal ubiquitin-associated (UBA) domain (ΔUBA), a mutant with deletion of both UBA and Mig6 homology (Mig6) domain (ΔMig6), and a mutant with deletion of UBA, Mig6 and the region particularly rich in proline residues, which we designated a proline-rich domain (ΔPRD). Each of these mutants was tagged with mCherry at the N-terminus. In the study we also took advantage of truncated Ack1, which lacks the C-terminal portion [including UBA, Mig6, PRD and clathrin binding domain (CBD)]. Using these constructs, we carried out a series of colocalisation studies with EGFR–GFP upon EGF stimulation. As shown in Fig. 2B, deletion of the UBA domain alone did not alter colocalisation between Ack1 and EGFR, which is similar to the full-length protein (~90%). In contrast, deletion of both UBA and Mig6 domains dramatically decreased this colocalisation (~43%). This is consistent with previous reports on the role of the Mig6 domain in the association between Ack1 and EGFR (Shen et al., 2007). Additional removal of the PRD does not have any further effect on the colocalisation with EGFR. However, the absence of the CBD, which is represented by tAck1, abolishes any remaining colocalisation (Fig. 2B). These data emphasise the importance of the Mig6 domain in this context and suggest that the association of Ack1 with clathrin also contributes to this colocalisation. Importantly, the UBA domain alone has no influence on the colocalisation of Ack1 and EGFR, suggesting that EGFR ubiquitylation is not required for colocalisation with Ack1.

### Ack1 partially localises to early endosomes upon EGF stimulation

We further analysed the subcellular localisation of Ack1 in the context of EGF signalling, because a precise localisation remains elusive, especially in pre-EGF conditions. Immunostaining of HeLa cells with anti-EEA1 (early endosome antigen 1) antibody reveals that in unstimulated cells (0 minutes EGF) mCherry–Ack1 poorly colocalised with EEA1, as manifested by a very low PCC (Fig. 3A). In contrast, at 15 minutes, and more noticeably at 30 minutes following EGF stimulation, Ack1 colocalisation with EEA1 significantly increased. These results are consistent with previous reports showing that Ack1 partially colocalises with EGFR on early endosomes (Shen et al., 2007; Grøvdal et al., 2008). However, recently, early endosomes have also been shown to be essential for autophagosome maturation (Razi et al., 2009; Tooze and Razi, 2009). Therefore, we also investigated whether Ack1 colocalises with ectopically expressed Rab5, which is a small GTPase that localises mostly to early endosomes, but has also been found on autophagosomes and other structures (Stenmark, 2009). Similar to EEA1, in unstimulated cells colocalisation of Ack1 with Rab5 was low, represented by a very low PCC (supplementary material Fig. S2A); however, as with EEA1, there was an increase in colocalisation of Ack1 and Rab5 following EGF treatment (supplementary material Fig. S2A). Taken together, these results indicate that following EGF stimulation, Ack1 traffics through EEA1- and Rab5-positive compartments. However, these data also raise the potential connection between Ack1 and non-canonical degradative pathways.

### Ack1 localises to pre-autophagosomal structures upon EGF stimulation

Apart from the classical endo/lysosomal pathway, other non-canonical degradation pathways exist. We and others show that Ack1 partially localises to EEA1-positive compartments upon treatment with EGF (Shen et al., 2007; Grøvdal et al., 2008). Because early endosomes are required for autophagosomal maturation (Razi et al., 2009; Tooze and Razi, 2009), we investigated whether Ack is involved in the autophagosomal pathway. Atg (autophagy-related) proteins, which are essential for autophagy, are required for the initiation and maturation of autophagosomes (Mizushima et al., 2011). At the initial stages of autophagosome formation, when the isolation membrane is not yet enclosed, a multimeric complex of Atg16L, Atg5 and Atg12

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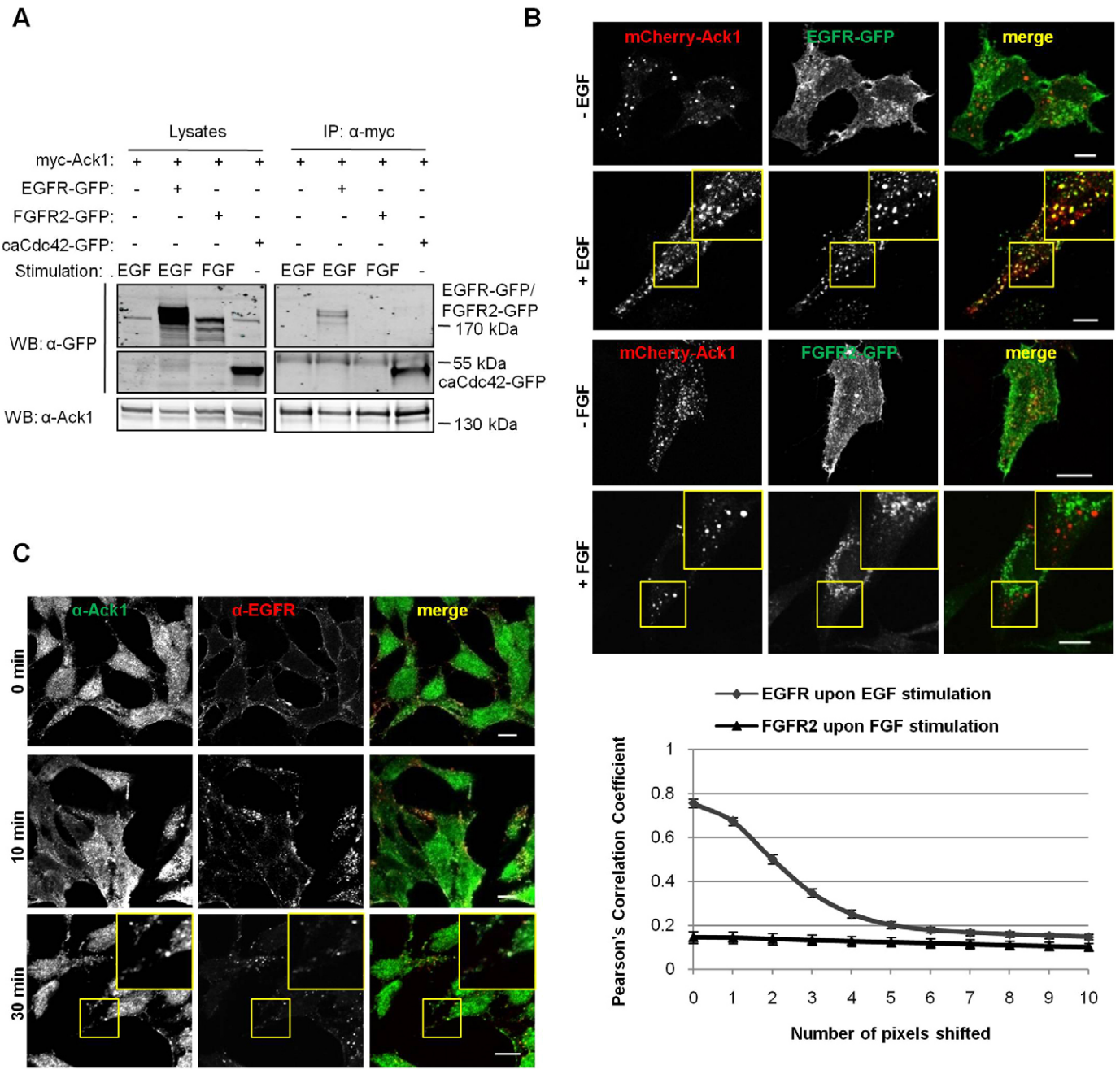


**Fig. 1. Ack1 interacts with EGFR, but not with FGFR.** (A) 293T cells transfected with Myc–Ack1 and EGFR–GFP, FGFR2–GFP or constitutive active Cdc42–GFP (caCDC42–GFP) were serum starved followed by stimulation for 20 minutes with EGF or FGF2 and heparin. Cells were lysed and subjected to immunoprecipitation with anti-Myc antibody. Western blot (WB): anti-GFP and anti-Ack1 antibodies. (B) HeLa cells transfected with mCherry–Ack1 and EGFR–GFP or FGFR2–GFP were serum starved following by stimulation with EGF or FGF2 with heparin for 30 minutes. Cells were fixed and imaged by confocal microscopy. The graph represents quantification of colocalisation using PCC of Ack1 and EGFR or FGFR2 upon stimulation with EGF or FGF2, respectively. (C) LNCaP cells were serum starved and stimulated with EGF for 10 or 30 minutes. Cells were fixed and immunostained with anti-Ack1 and anti-EGFR antibodies. Scale bars: 10 μm. Error bars represent s.e.m.

assembles, which dissociates upon membrane closure (Mizushima et al., 2011). We therefore examined whether Ack1 colocalises with Atg16L before or during EGF stimulation. As shown in Fig. 3B, in unstimulated cells Atg16L staining was diffuse; however, upon EGF stimulation, punctate Atg16-positive structures could be distinguished and Ack1 localised to these structures. The colocalisation of Ack1 and Atg16L was quantified (Fig. 3B); ~25% of Ack1 puncta were positive for Atg16L after 15 minutes of EGF treatment. This was significantly higher than random control regions not containing Ack1 (see the Materials and Methods) (Fig. 3B). Additionally, colocalisation of Ack1 and Atg16L was quantified with PCC, where an increase in colocalisation was observed 15 and 30 minutes after EGF treatment (Fig. 3B, bottom graph). Furthermore, endogenous Ack1 also colocalised with endogenous Atg16 (supplementary material Fig. S2B). Finally, immunoprecipitation of endogenous Atg16L resulted in co-precipitation of endogenous Ack1

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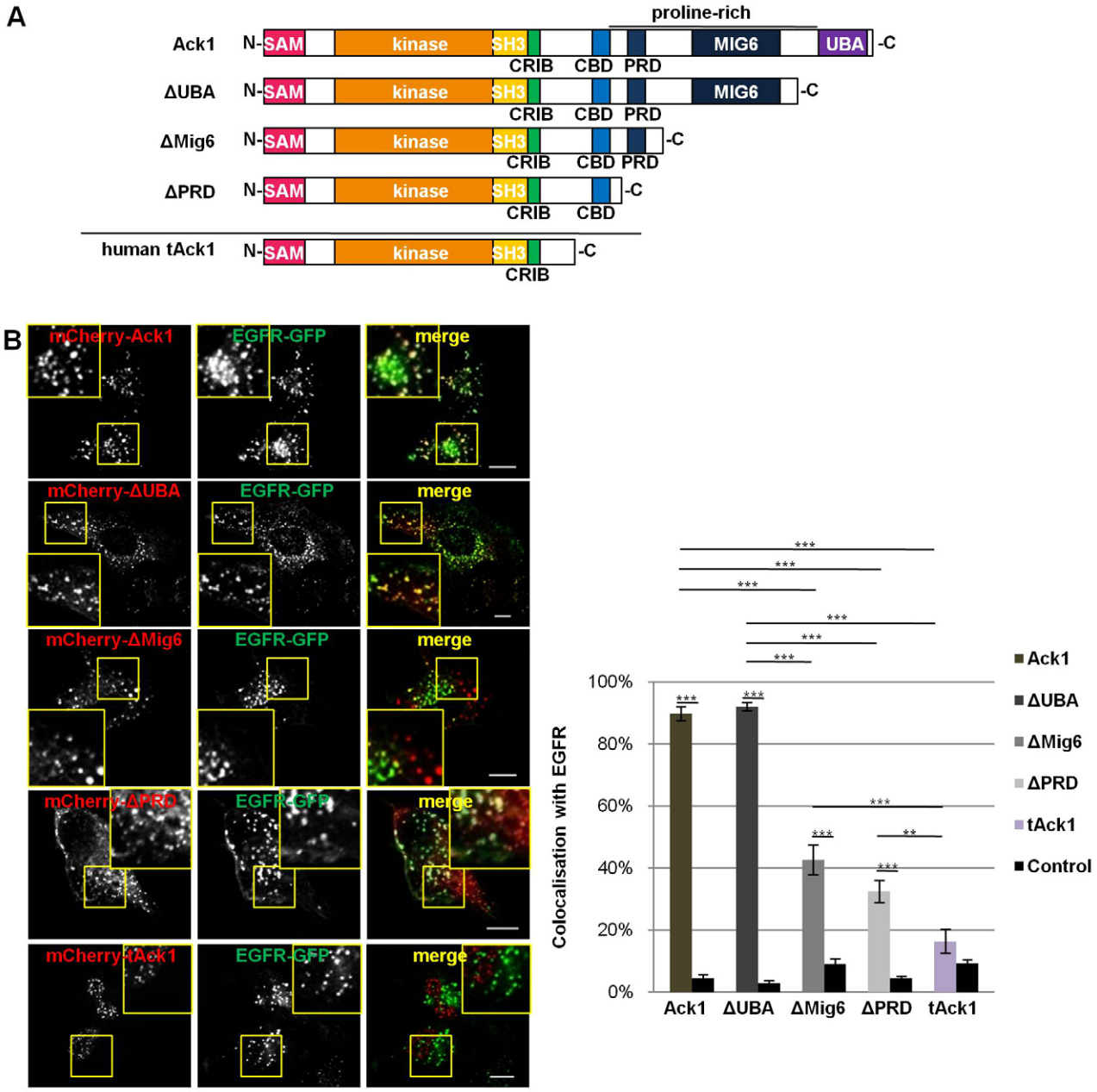


**Fig. 2. Mig6 and CBD of Ack1 regulate colocalisation with EGFR after EGF stimulation.** (A) mCherry-tagged C-terminal truncation mutants of Ack1 and tAck1. (B) HeLa cells transfected with EGFR–GFP and mCherry-tagged Ack1, tAck1 or Ack1 mutants were serum starved and stimulated with EGF for 30 minutes and fixed. For colocalisation as percentage, Ack1, tAck1 or the Ack1 mutant puncta were circled and colocalisation with EGFR was quantified. Scale bars: 10 µm. Error bars represent s.e.m. **0.01 >$P$>0.001 and ***$P$<0.001.

(Fig. 3C). Thus, these data show that Ack1 associates with Atg16L-positive structures, in particular upon EGF stimulation. Although growth factor signalling has been shown to inhibit autophagy (Wang and Levine, 2010; Mizushima et al., 2011), reports exist indicating a role for clathrin-mediated endocytosis in autophagosome formation (Ravikumar et al., 2010; Mari et al., 2011). Stimulation with EGF promotes clathrin-mediated endocytosis (Sorkin and Goh, 2009) and might therefore provide membranes for autophagosome formation.

### Ack1 localises within ubiquitin-rich compartments

Given that Ack1 is potentially involved in the autophagosomal pathway, which involves degradation of ubiquitylated cargo, and that Ack1 has previously been shown to bind both mono- and poly-ubiquitin, as well as ubiquitylated proteins (Shen et al., 2007), we analysed whether Ack1 colocalises with ubiquitin. Thus, we expressed GFP–Ack1 and HA–ubiquitin in HeLa cells. As shown in Fig. 4A, there was a strong colocalisation of Ack1 and ubiquitin. Importantly, we observed large ubiquitin-rich structures to which Ack1 localised, herein referred to as ‘ubiquitin-rich compartments’ (supplementary material Fig. S3A). As a negative control, we used truncated Ack1, which lacks the C-terminal portion, including the UBA domain (supplementary material Fig. S1B). We found that truncated Ack1 does not colocalise with ubiquitin (Fig. 4A). Consistently,

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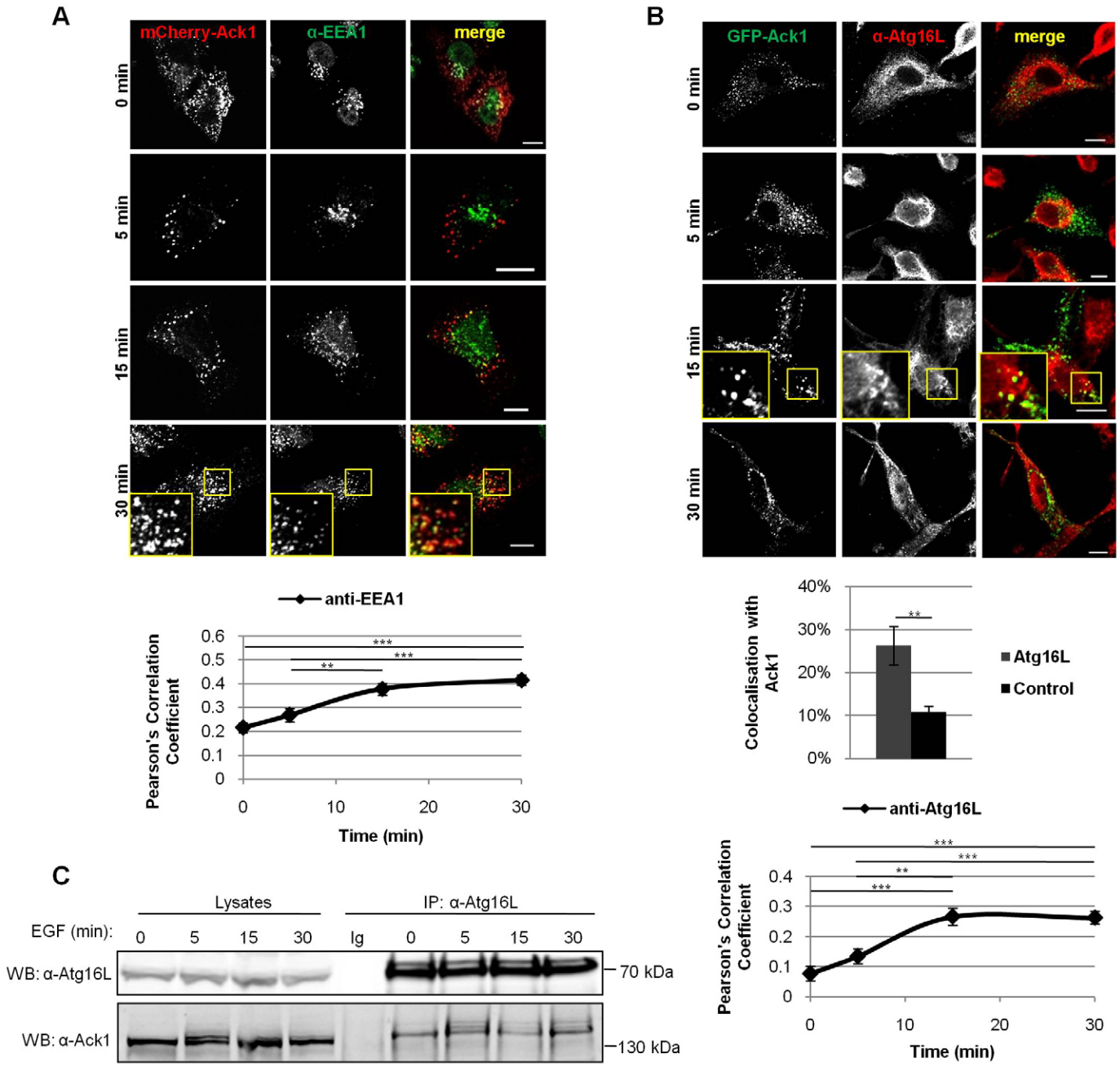


**Fig. 3. Ack1 partially colocalises with early endosomes and Atg16L-positive structures upon EGF stimulation.** (A) HeLa cells transfected with mCherry–Ack1 were serum starved and stimulated with EGF for indicated times and fixed. (B) HeLa cells transfected with GFP–Ack1 were serum starved and stimulated with EGF for indicated times, fixed and immunostained with anti-Atg16L antibody. For colocalisation as percentage, Ack1 puncta were circled. (C) LNCaP cells were serum starved and stimulated with EGF for indicated times, lysed and subjected to immunoprecipitation with anti-Atg16L antibody or with mouse IgG as a negative control. Scale bars: 10 μm. Error bars represent s.e.m. **0.01 $>P>$0.001 and ***$P<$0.001.

when performing immunoprecipitation of full-length and truncated Ack1, ubiquitin bound only full-length Ack1 and not tAck1 (Fig. 4B), and this is independent of EGF stimulation. Because the band detected by the antibody against GFP corresponds to the one detected by the HA antibody (Fig. 4B), and Ack1 has been shown to be ubiquitylated by Nedd4 ubiquitin ligases (Chan et al., 2009; Lin et al., 2010), we propose that this is ubiquitylated Ack1. Altogether, these results indicate that Ack1 is localised within ubiquitin-rich compartments and that the C-terminal portion of Ack1 is important for this localisation.

Next, we investigated whether EGFR colocalises with Ack1 within ubiquitin-rich compartments. As shown in Fig. 4C, upon EGF stimulation more than 90% of Ack1 puncta colocalised with EGFR, and nearly 70% of those were positive for ubiquitin. These results indicate that Ack1 and EGFR are localised within ubiquitin-rich compartments following EGF stimulation. Collectively, these data emphasise that Ack1 is involved in ubiquitin-dependent degradation of EGFR.

We further analysed which of the Ack1 domains regulate the colocalisation of Ack1 and ubiquitin. We found that high

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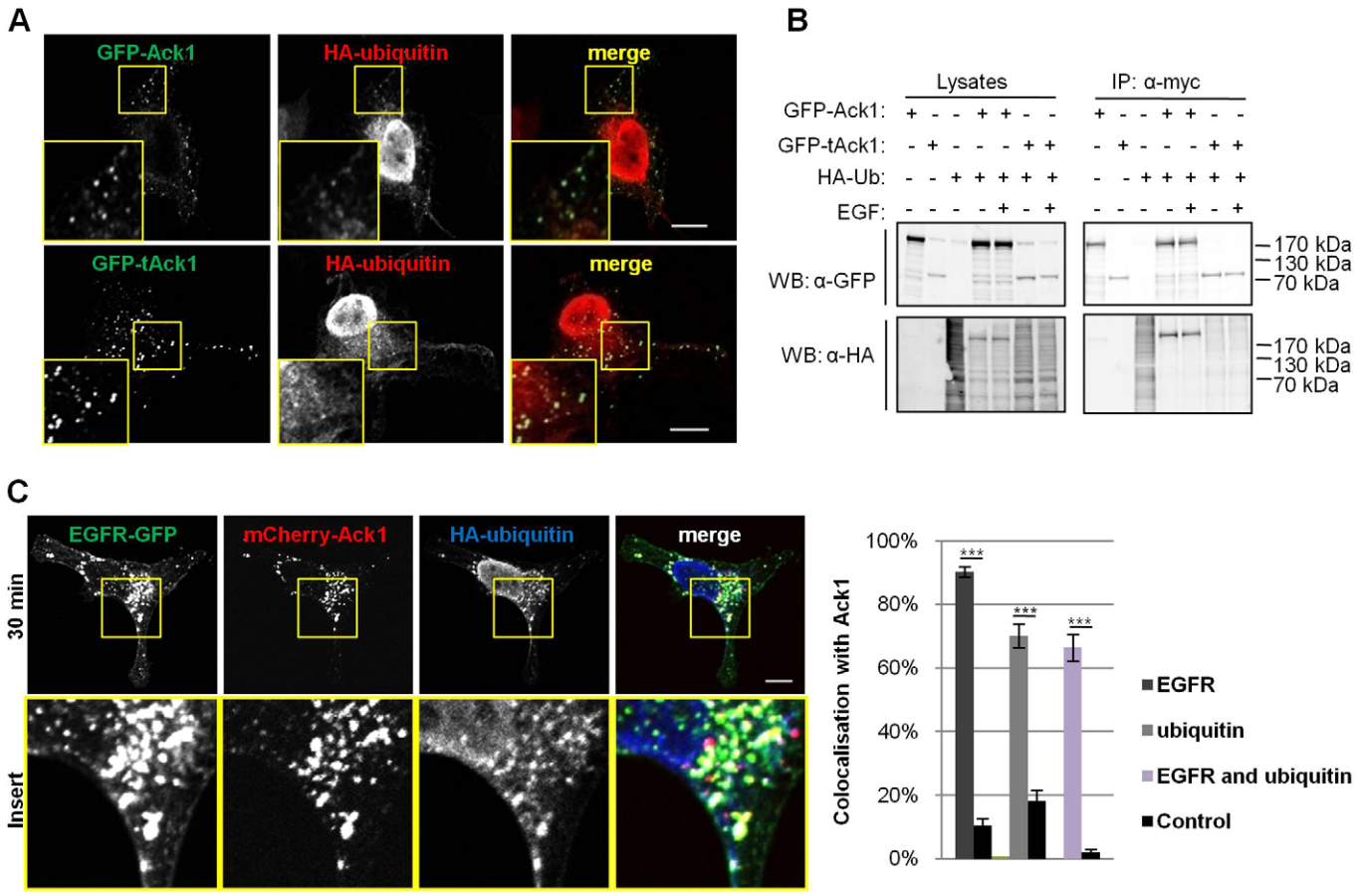


**Fig. 4. EGFR colocalises with Ack1 within ubiquitin-rich compartments upon EGF treatment.** (A) HeLa cells transfected with GFP–Ack1 or GFP–tAck1 and HA–ubiquitin were fixed and immunostained with anti-HA antibody. (B) 293T cells transfected with GFP–Ack1 or GFP–tAck1 and HA–ubiquitin were serum starved and stimulated with EGF for 30 minutes, lysed and subjected to pull-down with GFP–trap to precipitate GFP-tagged proteins. (C) HeLa cells transfected with mCherry–Ack1, EGFR–GFP and HA–ubiquitin were serum starved and stimulated with EGF for 30 minutes, fixed and immunostained with anti-HA antibody. For colocalisation as percentage, Ack1 puncta were circled. Nuclear localisation was excluded from the analysis. Scale bars: 10 μm. Error bars represent s.e.m. ***$P<0.001$.

colocalisation of Ack1 and ubiquitin (~80%) partially decreased following deletion of the UBA domain (~40–50%) (supplementary material Fig. S3B). We observed a further decrease in colocalisation following deletion of the PRD (20–30%). Finally, the colocalisation was nearly abolished in the case of tAck1. Importantly, the association with ubiquitin seemed to be independent of EGF treatment, and there was no difference in colocalisation before and after EGF stimulation (supplementary material Fig. S3B). These data indicate that the UBA domain, the PRD and CBD all contribute to the association between Ack1 and ubiqutin and/or ubiquitylated proteins.

### Ack1 interacts and colocalises with p62/SQSTM1, an autophagy receptor, in unstimulated cells

To further identify the ubiquitin-rich compartments where Ack1 is localised, we considered ubiquitin-rich protein aggregates, the formation of which is a common phenomenon in non-canonical degradative pathways (Kraft et al., 2010). p62/SQSTM1 and NBR1 are ubiquitin-binding proteins that have been proposed to act as cargo receptors in the process of autophagy (Lamark et al., 2009). We showed previously that p62 and NBR1 associate with Spred2, a signalling inhibitor, to promote degradation of RTKs, whereas NBR1 on its own inhibits RTKs degradation (Mardakheh et al., 2009; Mardakheh et al., 2010). Here we show that Ack1 highly colocalises with p62/SQSTM1 in unstimulated cells (Fig. 5A). This is represented by a high PCC, which gradually decreases upon pixel movement. Interestingly, this colocalisation decreases upon EGF treatment (Fig. 5A), suggesting that EGF stimulation negatively influences the association between Ack1 and p62/SQSTM1.

In addition to our colocalisation studies, we also show that endogenous p62/SQSTM1 co-precipitates with endogenous Ack1 in unstimulated LNCaP cells, and this interaction decreases following EGF stimulation (Fig. 5B). The interaction is also preserved in cells treated with bafilomycin A1, which inhibits lysosomal acidification and hence degradation (Fig. 5B). This data suggests that Ack1 and p62/SQSTM1 may be interacting during fusion of autophagosomes with lysosomes, a process sensitive to bafilomycin treatment (Yamamoto et al., 1998).

### p62/SQSTM1 promotes colocalisation of Ack1 and NBR1

We also examined the colocalisation of Ack1 and NBR1, another autophagy receptor. Previously we reported that NBR1 mainly localises to the limiting membranes of the late endosomes (Mardakheh et al., 2009). Here, we show that Ack1 interacted with NBR1 both in the presence and absence of ectopically

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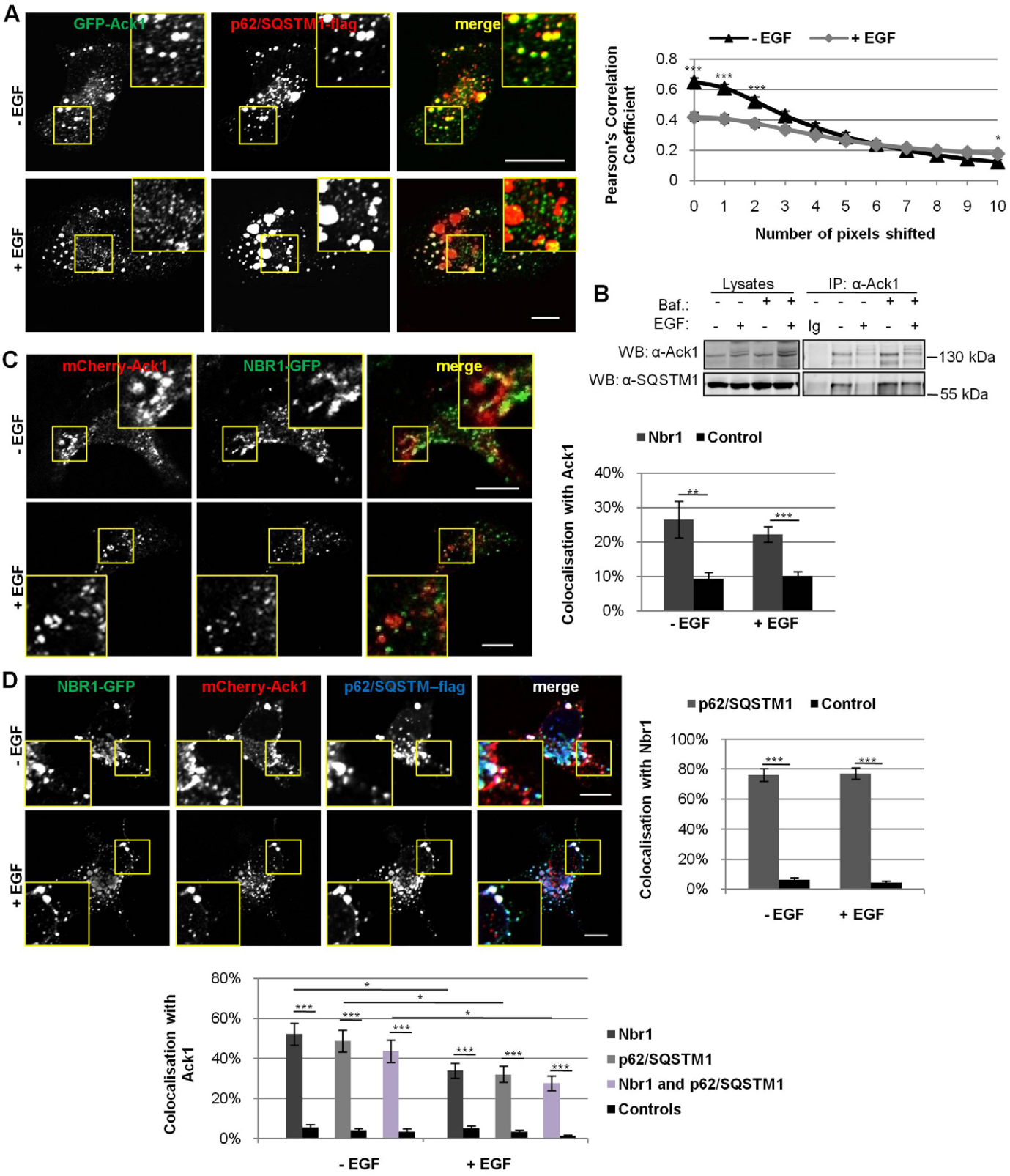


**Fig. 5. Ack1 interacts with an autophagy receptor p62/SQSTM1, and this interaction decreases upon EGF stimulation.** (A) HeLa cells transfected with GFP–Ack1 and p62–FLAG were serum starved and stimulated with EGF for 30 minutes, fixed and immunostained with anti-SQSTM1 antibody. (B) LNCaP cells were serum starved in the presence or absence of bafilomycin and stimulated with EGF for 10 minutes, lysed and subjected to immunoprecipitation with anti-Ack1 antibody or mouse IgG as a negative control. (C) HeLa cells transfected with mCherry–Ack1 and NBR1–GFP were serum starved and stimulated with EGF for 30 minutes and fixed. For colocalisation as percentage, Ack1 puncta were circled. (D) HeLa cells co-transfected with mCherry–Ack1, NBR1–GFP and p62–FLAG were serum starved and stimulated with EGF for 30 minutes, fixed and immunostained with anti-SQSTM1 antibody. For colocalisation as percentage, Ack1 puncta were circled and the colocalisation with NBR1 and p62/SQSTM1, or between NBR1 and p62/SQSTM1 within the Ack1 puncta, was quantified. Scale bars: 10 μm. Error bars represent s.e.m. *0.05>*P*>0.01, **0.01 >*P*>0.001 and ***$P<0.001$.

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expressed p62/SQSTM1 (supplementary material Fig. S4). We also show that Ack1 colocalised with NBR1, but the colocalisation is relatively low (~25%) and independent of EGF treatment (Fig. 5C). However, in the presence of ectopically expressed p62/SQSTM1, the colocalisation of NBR1 and Ack1 dramatically increased to ~52% in serum-starved cells (Fig. 5D). Furthermore, this colocalisation significantly decreased after EGF treatment (~34%). Importantly, there was a very strong colocalisation of NBR1 and p62/SQSTM1 (~80%) (Fig. 5D), suggesting that p62/SQSTM1 plays an active role in compartment maturation, promotes colocalisation of Ack1 and NBR1, and confers EGF sensitivity on this colocalisation.

We also found that upon EGF stimulation, internalised EGFR partially colocalised with Ack1 and p62/SQSTM1 (~20%), as shown in Fig. 6. This is consistent with our data showing that the colocalisation of Ack1 and p62/SQSTM1 decreased after EGF treatment. Interestingly, endogenous EGFR also partially colocalised with endogenous p62/SQSTM1, and this was not affected by knockdown of Ack1 (supplementary material Fig. S5A). Because we show that only a portion of EGFR present within the Ack1 puncta colocalised with p62/SQSTM1 (Fig. 6), it is possible that any difference in colocalisation in Ack1-knockdown cells is difficult to identify. Additionally, because knockdown of Ack1 was not complete (supplementary material Fig. S5B), it is possible that the remaining Ack1 is sufficient to mediate the colocalisation of EGFR and p62/SQSTM1. Altogether, these results indicate that serum deprivation promotes the interaction between Ack1 and p62/SQSTM1, whereas EGF stimulation might result in translocation of Ack1 away from the p62/SQSTM1 compartments. In summary, our data reveal a novel association between Ack1 and the autophagy receptors p62/SQSTM1 and NBR1.

### The C-terminal UBA domain regulates colocalisation of Ack1 and p62/SQSTM1

We further analysed the function of several Ack1 domains with respect to colocalisation with p62/SQSTM1. As shown in Fig. 7, in unstimulated cells Ack1 highly colocalised with p62/SQSTM1 (~55%) and this colocalisation partially decreased upon EGF stimulation (~30%). Thus, Ack1 colocalisation with p62/SQSTM1 post-EGF treatment is preserved, although to a much lower extent. Deletion of the UBA domain dramatically decreased the colocalisation between Ack1 and p62/SQSTM1 in unstimulated cells (~20%) and abolished EGF sensitivity. Importantly, deletion of the UBA domain did not have any effect on the colocalisation of Ack1 and NBR (supplementary material Fig. S6), indicating that the colocalisation of Ack1 and p62/SQSTM1 is highly specific. Further deletion of the Mig6 or PRD domains did not lead to any dramatic change in the colocalisation of Ack1 and p62/SQSTM1. However, any remaining colocalisation was abrogated in the case of tAck1. These data underscore the significance of the UBA domain in the association between Ack1 and p62/SQSTM1 and reveal that the presence of the UBA domain crucially confers the EGF sensitivity on this colocalisation.

### Silencing of Ack1 results in increased transient lysosomal localisation of EGFR

Because we identified an interaction between Ack1 and the autophagy receptors, we hypothesised that Ack1 prevents EGFR from trafficking through the canonical lysosomal pathway, and rather targets it into a non-canonical degradative pathway. Therefore, we investigated whether localisation of EGFR to lysosomes is affected in cells depleted of Ack1. Thus, LNCaP cells expressing GFP-tagged EGFR were treated with siRNA against Ack1 or with non-targeting RNAi control. Following serum starvation and incubation with LysoTracker, a fluorescent dye that stains lysosomes (Chazotte, 2011), the cells were imaged for 30 minutes after EGF stimulation. As shown in Fig. 8A, when compared with the control (supplementary material Movie 1), knockdown of Ack1 promotes increased transient colocalisation of EGFR and LysoTracker following EGF stimulation (supplementary material Movie 2). Silencing of Ack1 was verified by western blotting and quantified by real-time quantitative polymerase chain reaction as ~80%

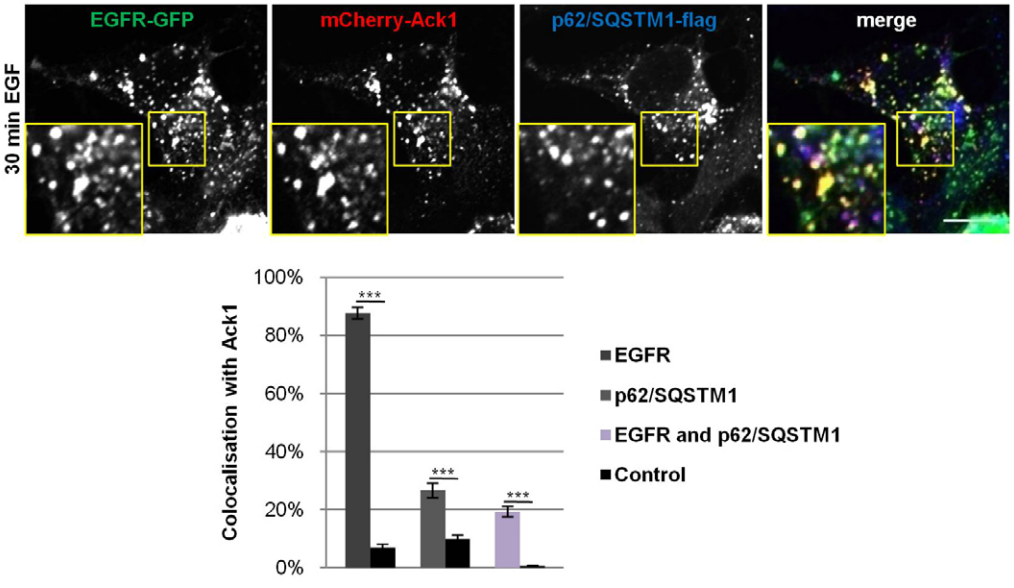


**Fig. 6. p62/SQSTM1 partially colocalises with Ack1 and EGFR upon EGF stimulation.** HeLa cells transfected with mCherry–Ack1, EGFR–GFP and p62–FLAG were serum starved and stimulated with EGF for 30 minutes, fixed and immunostained with anti-SQSTM1 antibody. For colocalisation as percentage, Ack1 puncta were circled and the colocalisation with EGFR and p62/SQSTM1, or between EGFR and p62/SQSTM1 within the Ack1 puncta, was quantified. Scale bars: 10 µm. Error bars represent s.e.m. ***$P<0.001$.

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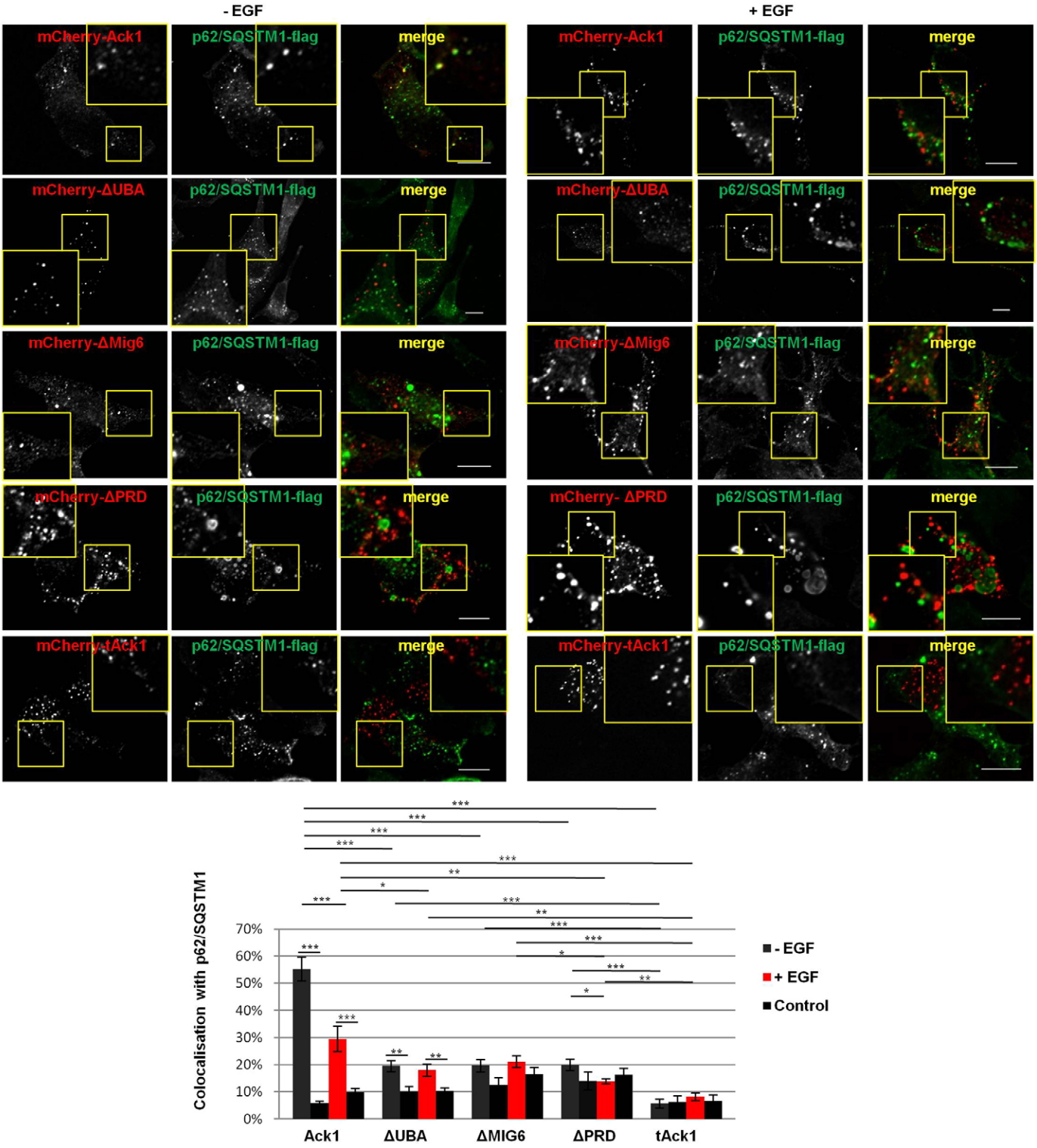


**Fig. 7. The UBA domain of Ack1 is essential for the association with p62/SQSTM1 and confers EGF sensitivity to this association.** HeLa cells transfected with p62–FLAG and mCherry-tagged Ack1, tAck1 or Ack1 mutants were serum starved and stimulated with EGF for 30 minutes, fixed and immunostained with anti-SQSTM1 antibody. For colocalisation as percentage, Ack1, tAck1 or the Ack1 mutant puncta were circled. Scale bars: 10 µm. Error bars represent s.e.m. *0.05>$P$>0.01, **0.01 >$P$>0.001 and ***$P$<0.001.

(supplementary material Fig. S7A,B). This observation therefore indicates that the presence of Ack1 delays EGFR recruitment to lysosomes. Interestingly, EGFR degradation was not affected by knockdown of Ack1 (supplementary material Fig. S8), indicating that the mechanism of EGFR degradation, rather than the EGFR degradation rate, is regulated by Ack1. Altogether, these data strongly support the proposed role of Ack1 in targeting EGFR into a non-canonical degradative pathway.



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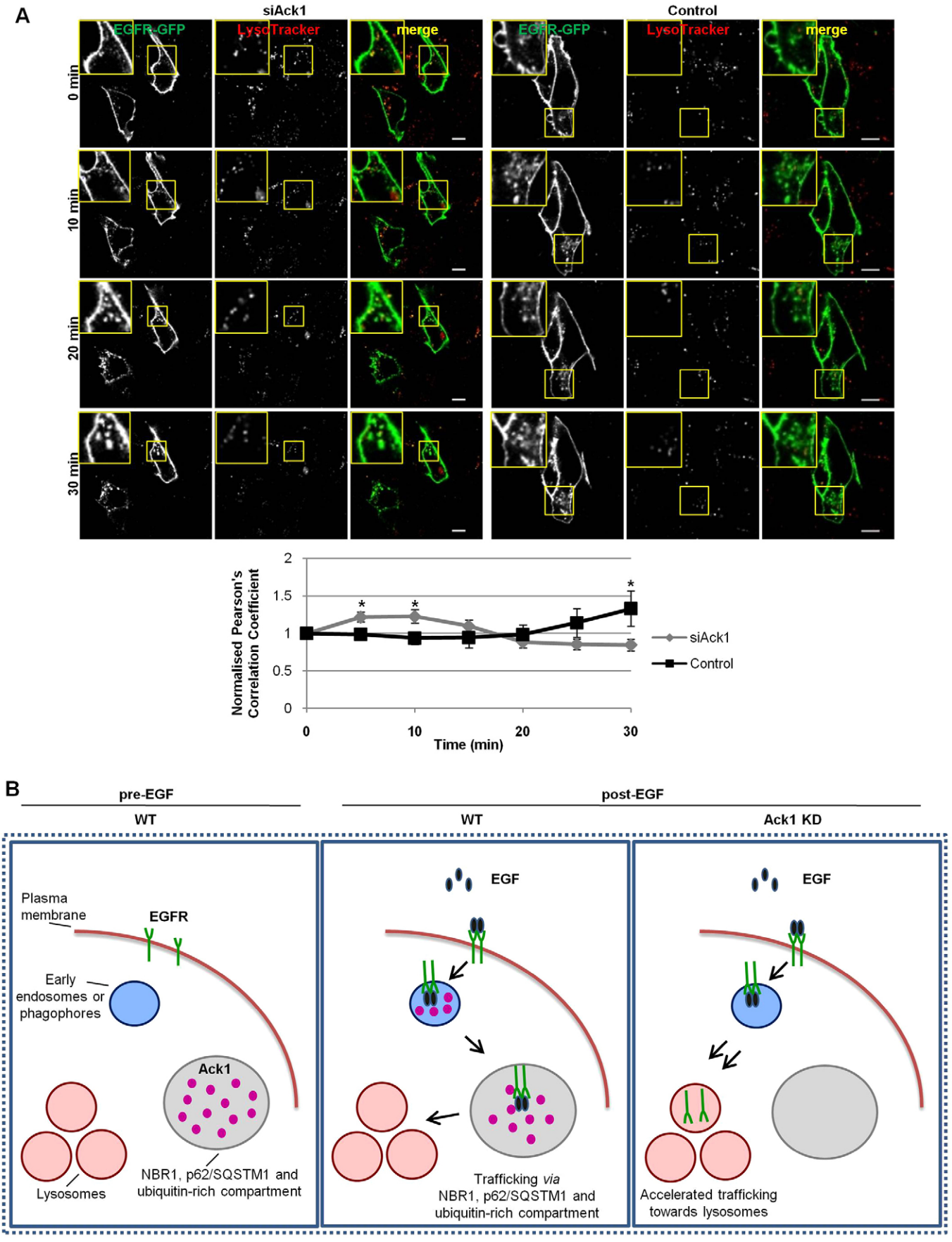


**Fig. 8.** See next page for legend.



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**Fig. 8. Silencing of Ack1 results in increased transient lysosomal localisation of EGFR.** (A) LNCaP cells transfected with EGFR–GFP and siRNA for Ack1 or non-silencing RNAi control were serum starved and incubated with LysoTracker Red for 30 minutes. Live-cell imaging was performed after 30 minutes of EGF stimulation. PCC was measured for the area within a cell (excluding the plasma membrane) at indicated times of EGF stimulation. For normalisation, PCC in unstimulated cells (0 min EGF) was set as 1, and the changes in PCC upon EGF stimulation were calculated relative to the PCC value in unstimulated cells. Scale bars: 10 μm. Error bars represent s.e.m. *$0.05>P>0.01$. (B) Schematic role of Ack1 in EGFR trafficking. Ack1 is predominantly present within p62/SQSTM1-rich compartments in serum-starved cells. Upon EGF stimulation, a portion of Ack1 translocates from p62/SQSTM1-rich compartments to early endosomes and diverts EGFR trafficking into a non-canonical degradative pathway. When Ack1 is knocked down, EGFR traffics through a canonical lysosomal pathway.

## DISCUSSION

Ack1 has been identified to regulate EGFR trafficking and degradation (Grøvdal et al., 2008), yet a precise function for Ack1 in this context has not been largely explored. Our work suggests a model for Ack1 function (Fig. 8B), in which Ack1 is localised within p62/SQSTM1 and ubiquitin-rich compartments in unstimulated cells; however, upon EGF stimulation, Ack1 localises away from p62/SQSTM1 to early endosomes to promote non-canonical trafficking of EGFR.

Previous studies suggest that Ack1 partially localises to early endosomes before (Prieto-Echagüe et al., 2010) and after EGF treatment (Shen et al., 2007; Grøvdal et al., 2008). However, we showed that colocalisation of Ack1 with the early endosomal marker EEA1 significantly increases after EGF stimulation (Fig. 3A) (Shen et al., 2007; Grøvdal et al., 2008). Following EGF treatment, Ack1 similarly colocalises with Rab5 (supplementary material Fig. S2A), which localises to early endosomes and other structures, including autophagosomes (Stenmark, 2009). It has been shown that the fusion of early endosomes with autophagosomes is required for autophagosome maturation (Razi et al., 2009). Therefore, the colocalisation with EEA1 led us to assess an autophagosomal localisation for Ack1. We found that following EGF stimulation, Ack1 partially colocalised with Atg16L (Fig. 3B,C), a protein that is essential for early stages of autophagosome formation (Matsushita et al., 2007). Thus, we propose that Ack1 localises to both early endosomes and autophagosomes upon stimulation with EGF.

Ack1 partially colocalises with clathrin and α-adaptin in cells at steady state (Teo et al., 2001). Furthermore, Ack1 has been detected by electron microscopy on large reticular membrane compartments upon EGF stimulation (Grøvdal et al., 2008); however, to our knowledge Ack1 localisation in serum-starved cells has not been reported. Therefore, we were interested in a precise subcellular localisation for Ack1, in particular upon serum starvation. Previously, it has been shown that Ack1 binds ubiquitin and ubiquitylated proteins (Shen et al., 2007) and in cells depleted of dynamin, Ack1 exhibits increased phosphorylation and binding of ubiquitylated proteins (Shen et al., 2011). Therefore, we examined the association between Ack1 and ectopically expressed ubiquitin, and found that Ack1 binds and colocalises with ubiquitin independent of EGF stimulation (Fig. 4B,C and supplementary material Fig. S3B). Because ubiquitylation regulates protein degradation (Kraft et al., 2010), we considered subcellular compartments rich in ubiquitylated proteins that are targeted for degradation. p62/SQSTM1 has been shown to act as an autophagy receptor and localise to ubiquitylated protein aggregates (Kraft et al., 2010). Here, we show that Ack1 colocalises with p62/SQSTM1 in unstimulated cells, and this colocalisation decreased following EGF stimulation (Fig. 5A). This is verified by biochemical studies, where we observed an interaction between endogenous Ack1 and p62/SQSTM1, which also decreases after EGF treatment (Fig. 5B). Therefore, we propose that in unstimulated cells Ack1 mainly localises to p62/SQSTM1- and ubiquitin-rich compartments.

In addition to our studies on p62/SQSTM1, we also observed colocalisation of Ack1 and NBR1, another autophagy receptor (Lamark et al., 2009). In this case, however, the colocalisation was not as striking as with p62/SQSTM1 (~25%) and was insensitive to EGF treatment (Fig. 5C). This strongly emphasises the specificity of the association between Ack1 and p62/SQSTM1. Importantly, colocalisation of Ack1 and NBR1 dramatically increased in the presence of ectopically expressed p62/SQSTM1 (~50%) (Fig. 5D), indicating that p62/SQSTM1 positively influences the colocalisation of Ack1 and NBR1. Although HeLa cells express endogenous p62/SQSTM1 (Pankiv et al., 2007), it might not be sufficient to affect the localisation of ectopically expressed proteins. Previously, we reported that NBR1 is a late endosomal protein that partially localises to autophagosomes, and that its late endosomal and autophagosomal localisation are independent (Mardakheh et al., 2009; Mardakheh et al., 2010). We therefore propose that NBR1 and p62/SQSTM1, despite interacting (Kraft et al., 2010), are present within different subcellular compartments as a result of other roles that they play within the cells. Because p62/SQSTM1 and NBR1 interact with each other through their Phox and Bem 1 (PB1) domains (Johansen and Lamark, 2011), we suggest that this leads to an indirect colocalisation of Ack1 and NBR1 in the presence of the ectopically expressed p62/SQSTM1.

Our structure–function studies demonstrated that the UBA domain of Ack1 is essential for association with p62/SQSTM1 (Fig. 7). Deletion of the UBA domain dramatically decreased this association and desensitised it to EGF treatment. This finding is particularly relevant considering that the UBA domain of several autophagic receptors, including p62/SQSTM1 and NBR1, is crucial for their function because it recognises ubiquitylated cargo, leading to its autophagic clearance (Bjørkøy et al., 2005; Johansen and Lamark, 2011). We also show that knockdown of Ack1 resulted in accelerated trafficking of EGFR towards lysosomal compartments (Fig. 8A). This therefore indicates that the presence of Ack1 prevents EGFR from rapid translocation to lysosomes following EGF stimulation and suggests that Ack1 plays a role in mediating EGFR trafficking into a non-canonical degradative pathway. In this context, it is surprising that deletion of the UBA domain of Ack1 has no effect on its association with EGFR, suggesting that ubiquitylation of EGFR is dispensable for this association. We therefore propose that the association of EGFR with the Mig6 and clathrin-binding domains of Ack1, accompanied by Ack1 association with ubiquitin and p62/SQSTM, act as an underlying mechanism to the non-canonical trafficking of EGFR.

In summary, our studies identify a novel role for Ack1 in a non-canonical degradative pathway. We propose that Ack1 'shuttles' between the p62/SQSTM1 compartments and the canonical endocytic pathway, and prevents EGFR trafficking by the classical lysosomal pathway.

## MATERIALS AND METHODS

### Antibodies and reagents

Anti-GFP (D5.1), anti-HA-tag (C29F4), anti-EEA1 and anti-EGFR polyclonal and monoclonal (D38B1) antibodies were purchased from

Cell Signaling. Anti-ACK (A-11 and C20) and anti-EGFR (R-1) were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Myc (clone 9E10) was from Roche Diagnostics (Mannheim, Germany). Anti-SQSTM1 (M01) (clone 2C11) was purchased from Abnova (Taipei City, Taiwan), anti-Rab11 from Invitrogen (Camarillo, CA), anti-Atg16L from MBL International (Woburn, MA), anti-LBPA (6C4) was from Echelon (Salt Lake City, UT) and anti-α-tubulin from Sigma (St Louis, MO). Alexa-Fluor-conjugated secondary antibodies for immunostaining were purchased from Invitrogen. Infrared-dye-conjugated secondary antibodies for western blotting as well as the Quick Western Kit were purchased from Li-Cor (Lincoln, NE). Mouse IgG was purchased from Santa Cruz, Lysotracker Red DND-99 from Invitrogen. Small interfering RNA (siRNA) against TNK2 and non-targeting iRNA control were purchased from Dharmacon (Lafayette, CO). EGF, heparin and poly-D-lysine were from Sigma and bafilomycin A1 from Merck Millipore (Darmstadt, Germany). The FGF2 was made in-house (Anderson et al., 1998). Briefly, the protein (155 amino acids; 18 kDa) was expressed in *E.coli* from the bacterial expression vector pFC80 (provided by Antonella Isacchi, Pharmacia & Upjohn, Milan, Italy) and purified by heparin-column chromatography.

### Plasmids

The murine Ack1 isoform 2 (UniProt: O54967-2) (1008 aa) N-terminal Myc-tagged in pcDNA3 vector and GFP-tagged in pEGFP-C1 vector were kindly provided by Wannian Yang (Geisinger, Danville, PA). For mCherry–Ack1, the open reading frame (ORF) was subcloned into pmCherry-C1 vector (Clontech, Mountain View, CA). The ORF of human Ack1 isoform 2 (truncated Ack1) (UniProt: Q07912-2) in a Gateway (Invitrogen) pDONR vector (Open Biosystems, Huntsville, AL) was subcloned into GFP–pcDNA3, Myc–PRK5 and pmCherry–C1 vectors. Human hFGFR1-pcDNA3.1 was a gift from Pamela Maher (The Scripps Research Institute, CA); human FGFR2-pEGFP-N2 was provided by John Ladbury (University of Texas M. D. Anderson Cancer Center, Houston, TX). EGFR-pEGFP-N1 was provided by Alexander Sorkin (University of Colorado, Aurora, CO), pcDNA-3 L61-Cdc42-GFP encoding GFP-tagged constitutively active Cdc42 (caCdc42) was provided by Neil Hotchin (University of Birmingham, Birmingham, UK). Rab5-EGFP was provided by Alexandre Benmerah (Cochin Institute, Paris, France). Ubiquitin–HA fusion in pMT123 vector was provided by Ronald Hay, University of St Andrews, UK). FLAG-p62/SQSTM1 in pcDNA3.1 vector was provided by Robert Layfield (University of Nottingham, Nottingham, UK).

### Ack1 C-terminal truncation mutants

C-terminal truncations of murine Ack1 isoform 2 were generated by PCR from a full-length construct using forward and reverse primers with *Eco*RI and *Bam*HI digestion sites, respectively. The linear products were digested with *Eco*RI and *Bam*HI restriction enzymes (New England Biolabs, Ipswich, MA) and ligated with pmCherry-C1 vector using T4 ligase (New England Biolabs). The following primers were used: forward primer for all mutants, TGAGTCCGTAGAATTCGATGCAGCCGGAG-GAGGGA and reverse primers for ΔUBA mutant (1–910 aa) TAGCCTAAGTGGATCCTCATCTGACGGTGGCAGT, ΔMIG6 mutant (1–680 aa) TAGCCTAAGTGGATCCTCAGGGCATCTGCGCCTG, ΔPRD mutant (1–610 aa) TAGCCTAAGTGGATCCTCACCGTGTGGGGCTCTG.

### Cell culture and transfection

Human embryonic kidney 293T (293T) and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS with 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine at 37℃ with 5% $CO_2$. LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% FBS with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, with addition of 2 mM L-glutamine at 37℃ with 5% $CO_2$. 293T and HeLa cells were transfected with GeneJuice Transfection Reagent (Novagen, Billerica, MA) and LNCaP cells with Lipofectamine 2000 (Invitrogen), according to the manufacturers' instructions. Cells were incubated for further 48 hours after transfection to allow for protein expression. Upon cell lysis, protein concentration in cell lysates was determined by Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Cell lysates were adjusted to the same protein concentration per experiment.

### Cell treatment

When indicated, cells were starved for 4 hours in medium without serum followed by stimulation with FGF2 (20 ng/ml) and heparin (10 μg/ml), or with EGF (100 ng/ml for HeLa and 293T cells and 20 ng/ml for LNCaP cells) for the indicated times. Cells were incubated with bafilomycin A1 (400 nM) for 4 hours prior to stimulation. In the case of lysosomal staining, cells were pre-incubated with LysoTracker Red (100 nM) for 30 minutes, washed twice with ice-cold PBS and placed in cell imaging medium (10 μM HEPES-HBSS, pH 7.4) for live-cell imaging.

### Immunoprecipitation and western blotting

Immunoprecipitation was performed using Protein-G–Sepharose beads (Sigma), Dynabeads (Invitrogen) or GFP–Trap (ChromoTek, Planegg-Martinsried, Germany) as indicated. For antibody crosslinking, Dynabeads conjugated with anti-Ack1 antibody were incubated with dimethyl pimelidate dihydrochloride (Sigma) in triethanolamine (pH 8.2) (Sigma) following by a glycine wash (pH 3.0) (Fishers Scientific, Fair Lawn, NJ). Immunoblots were imaged using Odyssey Application Software version 3.0 with the Odyssey Imaging System (Li-Cor).

### Immunostaining and cell imaging

Cells were plated onto coverslips 24–48 hours before immunostaining. In the case of LNCaP cells, coverslips were additionally pre-coated with poly-D-lysine (0.01 mg/ml) (Sigma) to enable cell attachment to coverslips. Cells were washed twice with ice-cold PBS and fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) or −20℃ methanol (for anti-Atg16L antibody). PFA-fixed cells were permeabilised with ice-cold 0.1% Triton X-100 (Sigma) for 5 minutes or 0.2% Triton X-100 for 3 minutes. For live-cell imaging, cells were plated onto a dish with a glass coverslip bottom (MalTek, Ashland, MA) in cell-imaging medium at 37℃. Images were acquired using a Nikon A1R confocal microscope with a 60× oil objective (N.A. 1.49) and analysed with Nikon NIS Elements software.

### Image quantification

For assessing colocalisation with the Pearson's correlation coefficient (PCC), a line was drawn around a cell, and the correlation between two channels (e.g. green and red) was measured as PCC. In the case of additional quantification with pixel shift, one channel (e.g. green) was shifted one pixel at time with reference to another channel (e.g. red), up to ten pixels. The gradual decrease in PCC was considered as genuine colocalisation. For assessing colocalisation as a percentage, puncta were circled and those that colocalised with another channel were counted and expressed as a percentage. As a negative control, the circles were moved into adjacent areas negative for fluorescence in a given channel, and the random colocalisation with another channel was quantified. The data were collected from at least three experiments, with minimum of three cells per experiment quantified.

### Real-time quantitative polymerase chain reaction (RT-qPCR)

LNCaP cells were transfected with siRNA against Ack1 or non-targeting RNAi control. 48 hours post-transfections cells were trypsinised and harvested by centrifugation, and RNA was isolated with RNeasy Mini kit (Qiagen, Hilden, Germany). RNA concentration was validated with NanoDrop, and 2 μg of RNA was subjected for synthesis of the cDNA with cDNA synthesis kit (Life Technologies, Carlsbad, CA). 25 ng of cDNA was added to the PCR mix with primers for Ack1 (TNK2) or 18S (Applied Biosystems). RT-qPCR was performed with ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The data were collected from three experiments.

### Statistical analysis

All the data were analysed with a two-tailed Student's *t*-test to compare the differences between two means. *$0.05>P>0.01$, **$0.01>P>0.001$ and ***$P<0.001$.

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### Competing interests
The authors declare no competing interests.

### Author contributions
S.J. designed and performed experiments, analysed data and wrote the paper. D.L.C. designed and performed experiments. J.Z.T. designed experiments and wrote the paper. J.K.H. designed experiments and wrote the paper.

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### Supplementary material
Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.136895/-/DC1

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