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

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

School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

Address to correspondence: Joshua Z. Rappoport, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK. Telephone: +44 (0) 121 414 5925. Email: j.rappoport@bham.ac.uk

Running title

Ack1 and non-canonical EGFR degradation

Keywords

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Summary

Growth factor signalling regulates multiple cellular functions and its misregulation has been linked to cancer development and progression. Ack1 (Activated Cdc42-associated kinase 1, TNK2), a non-receptor tyrosine kinase, has been implicated in trafficking and degradation of epidermal growth factor receptor (EGFR), yet the 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 EGF stimulation. These are proposed to be the isolation membranes during autophagosome formation. In addition we find that Ack1 colocalises and interacts with sequestosome 1 (p62/SQSTM1), a receptor for selective autophagy, via a ubiquitin associated domain and this interaction decreases upon EGF treatment, 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, Ack1 knock-down results in accelerated lysosomal localisation of EGFR upon EGF treatment. Structure-function analyses of a panel of Ack1 deletion mutants have revealed key mechanistic aspects of these relationships. The Mig6-homology domain and clathrin binding domain both contribute to the colocalisation with EGFR, whereas the UBA domain is critical for the 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.
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 dimerization, 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, EGFR endocytosis, incorporation into multi-vesicular bodies of late endosomes and lysosomal degradation are essential for signal attenuation and uncontrolled EGFR signalling has been found in different cancer types (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/TNK2) is a non-receptor tyrosine kinase that has been proposed to regulate EGFR trafficking (Grovdal et al., 2008), yet the precise mechanistic roles of Ack1 in this context remain elusive. High levels of Ack1 expression resulted in inhibition of EGFR degradation (Grovdal et al., 2008), possibly due to the disruption of endocytic machinery as a consequence of clathrin sequestration (Teo et al., 2001). Additionally, Ack1 down-regulation has also been shown to inhibit EGFR degradation and increase recycling, and Ack1 has been proposed to regulate endosomal sorting of EGFR into inner vesicles of multi vesicular bodies (Grovdal et al., 2008). Interestingly, an 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 is one of the major degradative pathways within the cell, which eliminates ubiquitylated protein aggregates and organelles. Formation of protein aggregates has been suggested to be mediated by autophagy
receptors, which have been shown to 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), e.g. EGF deprivation in mammary epithelial cells
(Fung et al., 2008).

Recently, we reported that NBR1 functions in RTK degradation (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, another
autophagic receptor (Lamark et al., 2009), and this interaction decreases upon EGF
stimulation. The UBA domain strongly regulates the association between Ack1 and
p62/SQSTM1, but not NBR1. Conversely, EGF stimulation results in Ack1 localisation to
Atg16-positive structures, which are likely to be pre-autophagosomal isolation membranes
(Matsushita et al., 2007; Mizushima et al., 2011). Furthermore, Ack1 silencing 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 via its association with
autophagic receptors p62/SQSTM1 and NBR1, which influences 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; Grovdal 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 FGF treatment (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 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 emphasize 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 post-EGF treatment (supplementary material Fig. S1C), indicating that the C-terminal fragment is essential for the Ack1-EGFR interaction. This is consistent with the studies showing that the Mig6 homology domain within the C-terminus of Ack1 mediates this interaction (Shen et al., 2007).

In order to study the physiological relevance of this interaction, 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, the colocalisation between endogenous Ack1 and EGFR can 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.

Mig6-homology domain (Mig6) and clathrin binding domain (CBD) 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 directly binds 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 the 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 does not alter the colocalisation between Ack1 and EGFR, which is similar to the full-length protein (~90%).
In contrast, deletion of both UBA and Mig6 domains dramatically decreases this colocalisation (~43%). This is consistent with the previous reports on the role of the Mig6 domain in the association between Ack1 and EGFR (Shen et al., 2007). Additional removing of the PRD does not have any further effects on the colocalisation with EGFR. However, the absence of the clathrin binding domain, which is represented by tAck1, abolishes any remaining colocalisation (Fig. 2B). These data emphasize the importance of the Mig6 domain in this context and suggest that Ack1 association with clathrin also contributes to this colocalisation. Importantly, the UBA domain alone has no influence on the colocalisation between Ack1 and EGFR suggesting that the EGFR ubiquitylation may not be 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, as a precise localisation remains elusive, in particular in pre-EGF conditions. Immunostaining of HeLa cells with an α-EEA1 (early endosome antigen 1) antibody reveals that in unstimulated cells (0 minutes EGF) mCherry-Ack1 poorly colocalises with EEA1, as manifested by a very low PCC (Fig. 3A). In contrast, at 15 and more noticeably at 30 minutes following EGF stimulation, Ack1 colocalisation with EEA1 significantly increases. These results are consistent with previous reports showing that Ack1 partially colocalises with EGFR on early endosomes (Shen et al., 2007; Grovdal 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 Ack1 colocalisation with Rab5 is very low, represented by a very low PCC (supplementary material Fig. S2A); however, as with EEA1, there is an increase in colocalisation between 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, this data also raises 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
EGF treatment (Shen et al., 2007; Grovdal et al., 2008). As early endosomes have also been reported to be required for autophagosomal maturation (Razi et al., 2009; Tooze and Razi, 2009), we investigated whether Ack1 may be involved in the autophagosomal pathway. Atg (autophagy-related) proteins, essential for autophagy, are required for initiation and maturation of autophagosomes (Mizushima et al., 2011). At the initial stages of the autophagosome formation, when the isolation membrane is not yet enclosed, a multimeric complex of Atg16L, Atg5 and Atg12 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 demonstrates diffuse staining; however, upon EGF stimulation, punctate Atg16-positive structures can be distinguished and Ack1 localises to these structures. The colocalisation between Ack1 and Atg16L was quantified on the graph in Fig. 3B; approximately 25% of Ack1 puncta were positive for Atg16L following 15 minutes of EGF treatment. This was significantly higher than random control regions not containing Ack1 (see Materials and methods) (Fig. 3B). Additionally, the colocalisation between Ack1 and Atg16L was quantified with PCC, where an increase in colocalisation is observed at 15 and 30 minutes post-EGF treatment (Fig. 3B, bottom graph). Furthermore, endogenous Ack1 also colocalises with endogenous Atg16 (supplementary material Fig. S2B). Finally, immunoprecipitation of endogenous Atg16L resulted in co-precipitation of endogenous Ack1 (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). EGF stimulation promotes clathrin-mediated endocytosis (Sorkin and Goh, 2009) and may therefore provide membranes for autophagosome formation.

**Ack1 localises within ubiquitin-rich compartments**

Given that Ack1 may potentially be 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 is a very strong colocalisation between 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 find that truncated Ack1 does not colocalise with ubiquitin (Fig. 4A). Consistently, when performing immunoprecipitation of full-length and truncated Ack1, ubiquitin binds only full-length and not with tAck1 (Fig. 4B) and this is independent of EGF stimulation. Since the band detected by α-GFP antibody corresponds to the one detected by α-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 colocalise with EGFR, and nearly 70% of those are positive for ubiquitin. These results indicate that Ack1 and EGFR are localised within ubiquitin-rich compartments following EGF stimulation. Collectively, these data emphasize that Ack1 is involved in ubiquitin-dependent EGFR degradation.

We further analysed which of the Ack1 domains regulate the colocalisation between Ack1 and ubiquitin. We show that the high colocalisation between Ack1 and ubiquitin (~80%) partially decreases following deletion of the UBA domain (~40-50%) (supplementary material Fig. S3B). We observe further decrease in colocalisation following deletion of the PRD (20-30%). Finally, the colocalisation is nearly abolished in the case of tAck1. Importantly, the association with ubiquitin seems to be independent of EGF treatment, and there is 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 ubiquitin and/or ubiquitylated proteins.

**Ack1 interacts and colocalises with p62/SQSTM1, an autophagic 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 the 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 interaction 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 between Ack1 and NBR1**

We also examined the colocalisation between 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 interacts with NBR1 both in the presence and absence of ectopically expressed p62/SQSTM1 (supplementary material Fig. S4). We also show that Ack1 colocalises 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 between NBR1 and Ack1 dramatically increases to ~52% in serum starved cells (Fig. 5D). Furthermore, this colocalisation significantly decreases post-EGF treatment (~34%). Importantly, there is a very strong colocalisation between NBR1 and p62/SQSTM1 (~80%) (Fig. 5D), suggesting that p62/SQSTM1 plays an active role in compartment maturation, promotes colocalisation between Ack1 and NBR1, and confers EGF sensitivity on this colocalisation.

We also find that upon EGF stimulation, internalised EGFR partially colocalises with Ack1 and p62/SQSTM1 (~20%), shown in Fig. 6. This is consistent with our data showing that the colocalisation between Ack1 and p62/SQSTM1 decreases post-EGF treatment. Interestingly, endogenous EGFR also partially colocalises with endogenous p62/SQSTM1, and this is not affected by Ack1 knock-down (supplementary material Fig. S5A). Since we show that only a portion of EGFR present within the Ack1 puncta colocalises with p62/SQSTM1 (Fig. 6), it is
possible that any difference in colocalisation in Ack1 knock-down cells is difficult to identify. Additionally, since Ack1 knock-down is not complete (supplementary material Fig. S5B), it is possible that the remaining Ack1 is sufficient to mediate the colocalisation between EGFR and p62/SQSTM1. Altogether, these results indicate that serum deprivation promotes the interaction between Ack1 and p62/SQSTM1, whereas EGF stimulation may result in Ack1 translocation 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 the colocalisation between 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 colocalises with p62/SQSTM1 (~55%) and this colocalisation partially decreases 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 decreases the colocalisation between Ack1 and p62/SQSTM1 in unstimulated cells (~20%) and abolishes EGF-sensitivity. Importantly, deletion of the UBA domain does not have any effect on the colocalisation between Ack1 and NBR (supplementary material Fig. S6), indicating that the colocalisation between Ack1 and p62/SQSTM1 is highly specific. Further deletion of the Mig6 or PRD domains does not lead to any dramatic change in the colocalisation between Ack1 and p62/SQSTM1. However, any remaining colocalisation is abrogated in the case of tAck1. These data underscore the extreme 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.

Ack1 silencing results in increased transient lysosomal localisation of EGFR

Since we identified an interaction between Ack1 and the autophagy receptors, we hypothesized that Ack1 may prevent EGFR from trafficking through the canonical lysosomal pathway, and rather targets it into a non-canonical degradative pathway. Therefore we investigated whether EGFR localisation 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 upon EGF stimulation. As shown in Fig. 8A, when compared to control (supplementary material Movie S1), Ack1 knock-down promotes increased transient colocalisation between EGFR and LysoTracker following EGF stimulation (supplementary material Movie S2). Ack1 silencing is verified by Western blotting and quantified by real-time quantitative polymerase chain reaction as ~80% (supplementary material Fig. S7A S7B). This observation therefore indicates that the presence of Ack1 delays EGFR recruitment to lysosomes. Interestingly, EGFR degradation is not affected by Ack1 knock-down (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.
Discussion

Ack1 has been identified to regulate EGFR trafficking and degradation (Grovdal 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-Echaguee et al., 2010) and after EGF treatment (Shen et al., 2007; Grovdal et al., 2008). However, we show that Ack1 colocalisation with the early endosomal marker EEA1 significantly increases post-EGF stimulation (Fig. 3A) (Shen et al., 2007; Grovdal 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 autophagosomal localization for Ack1. We find that following EGF stimulation, Ack1 partially colocalises with Atg16L (Fig. 3B and C), a protein critical for early stages of autophagosome formation (Matsushita et al., 2007). Thus, we propose that Ack1 localises to both early endosomes and autophagosomes upon EGF stimulation.

Ack1 partially colocalises with clathrin and α-adaptin in steady-state cells (Teo et al., 2001). Furthermore, Ack1 has been detected by electron microscopy on large reticular membrane compartments upon EGF stimulation (Grovdal et al., 2008); however, to our knowledge Ack1 localisation in serum-starved cells has not been reported. Therefore we were interested in a precise Ack1 subcellular localization, 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 exhibited increased phosphorylation and binding of ubiquitylated proteins (Shen et al., 2011). Therefore, we examined the association between Ack1 and ectopically expressed ubiquitin, with the observation that Ack1 binds and colocalises with ubiquitin independently of EGF stimulation (Fig. 4B and C and supplementary material Fig. S3B). Since ubiquitylation regulates protein degradation (Kraft et al., 2010), we considered subcellular compartments rich in ubiquitylated proteins 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 decreases following EGF
stimulation (Fig. 5A). This is verified by biochemical studies, where we observe an
interaction between endogenous Ack1 and p62/SQSTM1, which also decreases post-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 observe colocalisation between Ack1 and
NBR1, another autophagy receptor (Lamark et al., 2009). In this case, however, the
colocalisation is not as striking as with p62/SQSTM1 (~25%) and is insensitive to EGF
treatment (Fig. 5C). This strongly emphasizes the specificity of the association between Ack1
and p62/SQSTM1. Importantly, colocalisation between Ack1 and NBR1 dramatically
increases in the presence of ectopically expressed p62/SQSTM1 (~50%) (Fig. 5D), indicating
that p62/SQSTM1 positively influences the colocalisation between Ack1 and NBR1.

Although HeLa cells express endogenous p62/SQSTM1 (Pankiv et al., 2007), it may 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 localization are independent of each other (Mardakheh
et al., 2009; Mardakheh et al., 2010). We therefore propose that NBR1 and p62/SQSTM1,
despite interacting with each other (Kraft et al., 2010), are present within different subcellular
compartments due to other roles that they play within the cells. Since p62/SQSTM1 and
NBR1 interact with each other via their Phox and Bem 1 (PB1) domains (Johansen and
Lamark, 2011) we suggest that this leads to an indirect colocalisation between Ack1 and
NBR1 in the presence of the ectopically expressed p62/SQSTM1.

Our structure-function studies demonstrate that the UBA domain of Ack1 is critical for the
association with p62/SQSTM1 (Fig. 7). Deletion of the UBA domain dramatically decreases
this association and desensitizes it to EGF treatment. This finding is particularly relevant
considering that the UBA domain of several autophagic receptors, including p62/SQSTM1
and NBR1, is critical for their function as it recognizes ubiquitylated cargo leading to its
autophagic clearance (Bjorkoy et al., 2005; Johansen and Lamark, 2011). We also show that
Ack1 knock-down results in accelerated trafficking of EGFR toward 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 EGFR ubiquitylation 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 a 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 degradation via classical lysosomal degradation.
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 Signalling. Anti-ACK (A-11 and C20) and anti-EGFR (R-1) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-c-myc (clone 9E10) from Roche Diagnostics (Mannheim, Germany). Anti-SQSTM1 (M01) (clone 2C11) was purchased from Abnova Taipei City, Taiwan), anti-Rab11 from Invitrogen (Camarillo, CA, USA), anti-Atg16L from MBL International (Woburn, MA, USA). anti-LBPA (6C4) was from Echelon (Salt Lake City, UT, USA) and anti-α-tubulin from Sigma (Saint Louis, MO, USA). Alexa-Fluor-conjugated secondary antibodies for immunostaining were purchased from Invitrogen. Infrared dye-conjugated secondary antibodies for Western blotting as well as Quick Western Kit were purchased from Li-cor (Lincoln, NE, USA). 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, USA). 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 Dr. Antonella Isacchi, Pharmacia & Upjohn, Milan, Italy) and purified by heparin-column chromatography.

Plasmids

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

**Ack1 C-terminal truncation mutants**

C-terminal truncations of murine Ack1 isoform 2 were generated via PCR from a full-length construct using forward and reverse primers with EcoR1 and BamH1 digestion sites, respectively. The linear products were digested with EcoR1 and BamH1 restriction enzymes (New England Biolabs, Ipswich, MA, USA) and ligated with pmCherry-C1 vector using T4 ligase (New England Biolabs). The following primers were used: forward primer for all mutants: TGAGTCCGTTAGAATTCGATGCAGCCGGAGGAGGGA and reverse primers for ΔUBA mutant (1-910 a.a.) TAGCCTAAGTGGGATTCATCTGACGGTGACGT, ΔMIG6 mutant (1-680 a.a.) TAGCCTAAGTGGGATTCATCTGACGGTGACGT, ΔPRD MUTANT (1-610 a.a.) TAGCCTAAGTGGGATTCATCTGACGGTGACGT.

**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 °C with 5% CO₂. 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 °C with 5% CO₂. 293T and HeLa cells were transfected with GeneJuice Transfection Reagent (Novagen, Billerica, MA, USA) and LNCaP cells with Lipofectamine 2000 (Invitrogen), according to manufacturers’ instructions. Cells were incubated for further 48 hours post-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, USA) according to the manufacturer’s instruction. 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 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 cross-linking, Dynabeads conjugated with α-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, USA). Immunoblots were imaged via *Odyssey* Application Software version 3.0 with Odyssey Imaging System (Lico).

**Immunostaining and cell imaging**

Cells were plated onto coverslips 24-48 hours prior to 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, USA) or -20°C methanol (for α-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, USA) in cell imaging medium at 37 °C. Images were acquired via Nikon A1R confocal microscope using 60x oil objective (N.A. 1.49) and analysed with Nikon *NIS Elements* software.

**Image quantification**
For assessing colocalisation via 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 which 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 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 (Quiagen, 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, USA). 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, USA). The data were collected from three experiments.

Statistical analysis
All the data were analysed with two-tailed Student’s t-test to compare the differences between two means. * represents p value 0.05>p>0.01, ** represents 0.01>p>0.001 and *** represents p<0.001.
Acknowledgements

This work was supported by Cancer Research UK (C80/A10171). The Nikon A1R/TIRF microscope used in this study was obtained through the Birmingham Science City Translational Medicine Clinical Research and Infrastructure Trials Platform, with support from Advantage West Midlands. We thank Susan Brewer for cloning of the following constructs: mCherry-Ack1, myc-tagged and GFP-tagged truncated Ack1, generation the C-terminal truncation mutants of Ack1, and for bacterial expression and purification of FGF2. We also thank Professor Hing Leung (The Beatson Institute for Cancer Research, Glasgow, UK) for providing LNCaP cells.


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 following by 20 minutes stimulation with EGF or FGF2 and heparin. Cells were lysed and subjected to immunoprecipitation with α-myc antibody. Western blot (WB): α-GFP and α-Ack1 antibodies, (B) HeLa cells transfected with mCherry-Ack1 and EGFR-GFP or FGFR2-GFP were serum-starved following by 30 minutes stimulation with EGF or FGF2 with heparin. Cells were fixed and imaged via confocal microscopy. The graph represents quantification of colocalisation via PCC between 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 α-Ack1 and α-EGFR antibodies. Scale bars 10 µm. Error bars represent SEM.

Fig. 2. Mig6 and CBD of Ack1 regulate colocalisation with EGFR post-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 mutants’ puncta were circled and the colocalisation with EGFR was quantified. Scale bars 10 µm. Error bars represent SEM.

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 α-Atg16L antibody. Scale bar 10 µm. 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 IP with α-Atg16L antibody or with mouse IgG as a negative control. WB: α-Atg16L and α-Ack1. Scale bars 10 µm. Error bars represent SEM.

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 α-HA antibody, (B) 293T cells transfected with GFP-Ack1 or
GFP-tAck1 and HA-ubiquitin were serum-starved and stimulated with EGF for 30 min, lysed and subjected to pull-down with GFP-trap to precipitate GFP-tagged proteins. WB: α-GFP and α-HA (C) HeLa cells transfected with mCherry-Ack1, EGFR-GFP and HA-ubiquitin were serum starved and stimulated with EGF for 30 min, fixed and immunostained with α-HA antibody. For colocalisation as percentage, Ack1 puncta were circled. Nuclear localisation was excluded from analysis. Scale bars 10 µm. Error bars represent SEM.

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 min, fixed and immunostained with α-SQSTM1 antibody, (B) LNCaP cells were serum starved in the presence or absence of bafilomycin and stimulated with EGF for 10 min, lysed and subjected to IP with α-Ack1 antibody or mouse IgG as a negative control. WB: α-Ack1 and α-SQSTM1, (C) HeLa cells transfected with mCherry-Ack1 and NBR1-GFP were serum starved and stimulated with EGF for 30 minu 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 min, fixed and immunostained with α-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, has been quantified. Scale bars 10 µm. Error bars represent SEM.

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 min, fixed and immunostained with α-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, has been quantified. Scale bars 10 µm. Error bars represent SEM.

Fig. 7. The UBA domain of Ack1 is critical 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 min, fixed and immunostained with α-SQSTM1 antibody. For colocalisation as percentage, Ack1, tAck1 or the Ack1 mutants’ puncta were circled. Scale bars 10 µm. Error bars represent SEM.

**Fig. 8. Ack1 silencing 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 upon 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 relatively to the PCC value in unstimulated cells. Scale bars 10 µm. Error bars represent SEM. (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 away 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 via a canonical lysosomal pathway.

**Fig. S1.** (A) **Ack1 does not interact with FGFR1.** 293T cells transfected with myc-Ack1 and FGFR1 were in complete medium (n/a) or serum-starved and stimulated with FGF2 and heparin for 20 minutes. Cells were lysed and subjected to immunoprecipitation with α-FGFR1 antibody. WB: α-FGFR1 and α-Ack1 antibodies, (B) **Schematic domain structure of full-length and truncated Ack1.** (C) **Truncated Ack1 does not colocalise with EGFR post-EGF.** HeLa cells transfected with myc-tAck1 and EGFR-GFP were serum-starved and stimulated with EGF for 30 minutes, fixed and immunostained with α-myc antibody. Scale bars 10 µm.

**Fig. S2.** Ack1 partially colocalises with Rab5 post-EGF, and with Atg16. A) HeLa cells transfected with mCherry-Ack1 and GFP-tagged Rab5 were serum-starved and stimulated with EGF for various times. Cells were fixed and analysed via confocal microscopy. The graph represents the quantification of colocalisation between Ack1 and Rab5 at various times.
post-EGF, (B) LNCaP cells were serum-starved and stimulated with EGF for various times. Cells were fixed and immunostained with α-Ack1 and α-Atg16 antibodies, and analysed via confocal microscopy. Scale bars 10 µm. Error bars represent SEM.

Fig. S3. Ack1 localises within ubiquitin-rich compartments. HeLa cells transfected with mCherry-Ack1 and HA-ubiquitin were fixed and immunostained with α-HA antibody, (B) Several domains contribute to the colocalisation between Ack1 and ubiquitin. HeLa cells transfected with HA-ubiquitin and mCherry-tagged Ack1, tAck1 or Ack1 mutants were serum-starved and stimulated with EGF for 30 minutes. Cells were fixed, immunostained with α-HA antibody and imaged via confocal microscopy. The graph represents the quantification of colocalisation between Ack1, tAck1 or the Ack1 mutants and ubiquitin as in Fig. 2B. Scale bars 10 µm. Error bars represent SEM.

Fig. S4. Both p62/SQSTM1 and NBR1 interact with Ack1. 293T cells transfected with myc-Ack1, NBR1-GFP and p62/SQSTM1-flag were serum-starved and stimulated with EGF for 30 minutes. Cells were lysed and subjected to immunoprecipitation with α-myc antibody. WB: α-Ack1, α-GFP and α-SQSTM1 antibodies.

Fig. S5. Endogenous EGFR and p62/SQSTM1 partially colocalise in the presence and absence of Ack1. A) LNCaP cells transfected with siRNA against Ack1 or non-targeting RNAi control were serum-starved and stimulated with EGF for indicated times. The cells were fixed and immunostained with α-EGFR and α-SQSTM1 antibodies, B) LNCaP cells were transfected with siRNA against Ack1 or non-targeting RNAi control and lysed. WB: α-Ack1, α-tubulin.

Fig. S6. The association between Ack1 and NBR1 is not mediated by the UBA domain. HeLa cells transfected with mCherry-Ack1 or ΔUBA mutant and NBR1-GFP were serum-starved and stimulated with EGF for 30 minutes. Cells were fixed and imaged via confocal microscopy. The graph represents the quantification of colocalisation between Ack1 or ΔUBA mutant and NBR1 as in Fig. 2B. Scale bars 10 µm. Error bars represent SEM.
**Fig. S7. Ack1 silencing in LNCaP cells.** A) LNCaP cells were transfected with siRNA against Ack1 or non-targeting RNAi control and EGFR-GFP, and lysed. WB: α-Ack1, α-EGFR and α-tubulin. B) LNCaP cells were transfected with siRNA against Ack1 or non-targeting RNAi control. Total RNA was isolated following by the cDNA synthesis, and the levels of Ack1 cDNA were quantified in reference to ribosomal 18S cDNA. Error bars represent SEM.

**Fig. S8. EGFR degradation rate is not affected by Ack1 knock-down.** LNCaP cells transfected with siRNA against Ack1 or non-targeting RNAi control were serum-starved and stimulated with EGF for indicated times, then lysed. WB: α-Ack1, α-EGFR, α-tubulin. Densitometry analysis from five experiments was performed using Odyssey Imaging System (Li-cor). Error bars represent SEM.

**Movie S1 and Movie S2.** Ack1 silencing results in increased lysosomal localisation of EGFR. LNCaP cells transfected with EGFR-GFP and non-silencing RNAi control (Movie S1) or siRNA for Ack1 (Movie S2) were serum starved and incubated with LysoTracker Red for 30 minutes. Live-cell imaging was performed upon 30 minutes of EGF stimulation, one frame per minute.
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- 170 kDa
- 55 kDa
caCdc42-GFP
- 130 kDa