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# Somatic mutation in the ACK1 ubiquitin association domain enhances oncogenic signaling through EGFR regulation in renal cancer derived cells

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## ABSTRACT

Activated Cdc42-associated Kinase, ACK1, is a non-receptor tyrosine kinase with numerous interacting partners, including Cdc42 and EGFR. Gene amplification and overexpression of ACK1 were found in many cancer types such as those of the lung and prostate. Previously, we identified both somatic- and germ line missense mutations in the ACK1 coding sequence, by surveying 261 cancer cell lines and 15 control tissues. Here, we verified and characterized the non-synonymous mutation, ACK-S985N, located in the ubiquitin association domain of the protein. Both overexpression and silencing experiments in MCF7 and A498 cells, respectively, demonstrated a role of the ACK1 S985N mutation in enhancing cell proliferation, migration and anchorage-independent growth as well as the epithelial–mesenchymal transition. Further, we showed that the ACK1 S985N mutant is unable to bind ubiquitin, unlike the wild type kinase. This contributed to ACK1 protein stability and stabilized EGFR after EGF stimulation, thereby prolonging mitogenic signaling in cancer cells. In addition, the ACK1 S985N-EGFR interaction is enhanced, but not the ubiquitination of the receptor. Intriguingly, silencing of ACK1 in A498 cells sensitized the renal carcinoma cells to gefitinib, against which they are otherwise resistant.

The work demonstrates that other than gene amplification, a single somatic mutation in ACK1 can result in extended protein stability enabling the oncoprotein to exert its oncogenic function in tumor progression. It also provides a rationale to target ACK1 in combination with other chemotherapeutic drugs, such as EGFR inhibitors, to potentiate therapeutic action against resistant tumors.

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## 1. Introduction

Protein Tyrosine Kinases (PTKs) are important regulators of signaling pathways that control fundamental cellular processes such as proliferation, differentiation, migration or cell

survival. Various PTKs, in either wild type or mutated form, behave as potent oncoproteins and have been implicated in cancer initiation and progression.

ACK1, or Activated Cdc42-Associated Kinase, located on chromosome 3q, is a ubiquitously expressed non-receptor

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Abbreviations: ACK1, activated Cdc42-associated kinase 1; UBA, Ubiquitin association domain; EGFR, epidermal growth factor receptor; RCC, renal clear cell carcinoma; EMT, epithelial–mesenchymal transition; TNF, tumor necrosis factor; HSP60, heat shock protein 60. 1574-7891/\$ – see front matter © 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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tyrosine kinase cloned from a human brain cDNA library (Manser et al., 1993). It was first identified to bind to activated Cdc42, a small Ras GTPase via its CRIB domain (Manser et al., 1993). This interaction activates ACK1 via autophosphorylation which subsequently activates Dbl, a Rho guanine exchange factor (Kato et al., 2000) as well as p130<sup>cas</sup> (Modzelewska et al., 2006). Dbl in turn activates the Rho family of GTPases, resulting in cytoskeletal rearrangements. In the last few years, reports have shown that Rho family proteins play an important role in tumor progression (Rosel et al., 2008; Sequeira et al., 2008; Tang et al., 2008; Vega and Ridley, 2008). In addition to activated Cdc42, other ACK1 interacting partners include clathrin, Grb2, EGFR, ubiquitin and Nedd4-2 E3 ligase (Chan et al., 2009; Kato-Stankiewicz et al., 2001; Shen et al., 2007; Teo et al., 2001). Its association with clathrin has been proposed to be involved in receptor-mediated endocytosis (Shen et al., 2007). ACK1 is shown to bind EGFR in a Grb2-dependent manner and aids EGFR downregulation with its functional ubiquitin association domain (UBA) (Grovdal et al., 2008; Kato-Stankiewicz et al., 2001; Shen et al., 2007).

Recently, ACK1 has been implicated in cancer progression. Gene amplification of ACK1 was reported in several cancer types such as lung-, prostate- and ovarian tumors (van der Horst et al., 2005), while cDNA microarrays demonstrated an upregulation of ACK1 expression in gastric carcinoma (Wang et al., 2006). ACK1 is also associated with androgen-independent prostate cancer (Mahajan et al., 2007) and aids in tumor metastasis through its positive activation of p130<sup>cas</sup> which, localized to the focal adhesion sites, responses to integrin signaling and is involved in spreading of melanoma cells (Eisenmann et al., 1999). ACK1 also exerts an oncogenic function through tumor suppressor, Wwox phosphorylation, targeting it for polyubiquitination and subsequent degradation (Mahajan et al., 2005). In LnCaP and 4T1 xenograft models, ACK1-overexpressing cells give rise to aggressive metastatic tumors (Mahajan et al., 2007; van der Horst et al., 2005). Silencing of the ACK1 gene in Ras-transformed NIH3T3 cells on the other hand increases apoptosis (Nur et al., 2005).

Previously, we reported the detection of several novel somatic- and germ line mutations in the coding sequence of ACK1 of different cancer cell lines and control tissues (Ruhe et al., 2007). In this paper, we characterized two somatic mutations, A634 T and S985 N in KatoIII and A498 cells, respectively. A634 T is located in the newly established E3 ligase binding domain (Chan et al., 2009) while S985 N is found in the UBA domain. We rationalized that the location of the mutations may confer extended protein stability to the kinase, thereby unleashing an inherent oncogenic activity. Our data demonstrate that the S985 N mutation in ACK1 confers enhanced proliferative and migratory properties as well as anchorage-independent growth. Mechanistic studies revealed that the ACK1 S985 N mutation has lost ubiquitin interaction, which in turn, enhances kinase stability, delays ubiquitination and reduces downregulation of EGFR. Interestingly, silencing of ACK1 S985 N sensitizes renal carcinoma cells to gefitinib. These findings not only provide us with new insights into mechanisms that underlie ACK1-mediated oncogenesis but also highlight the possibility of combination therapy by targeting ACK1 in cancer cells that are previously resistant to EGFR inhibitors.

## 2. Material and methods

### 2.1. Cell culture and reagents

All cells used were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) with exception to A498 which was cultured in MEM supplemented with pyruvate, non-essential amino acid and FBS. Phoenix A cells were passaged in Hygromycin (300 µg/ml) and Diphtheria Toxin (1 µg/ml) for 2 weeks before use. Cells were maintained in 5% CO<sub>2</sub> at 37 °C. All reagents were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise stated. Myc-tagged ubiquitin and FLAG-tagged MOP1 are kind gifts of Dr. Yu and the pXJ-ACK1 wild type construct was generously provided by A/P Manser.

### 2.2. PCR and sequencing

Genomic DNA was isolated using QIAamp DNA mini kit (Qiagen, Alameda, CA) and total RNA was isolated using RNeasy<sup>®</sup> Mini kit (Qiagen) followed by reverse transcription to cDNA using SuperScript<sup>™</sup> III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Primers for amplification and sequencing of cDNA were as described previously (Ruhe et al., 2007). The primers for genomic DNA sequencing were as follows: A634 T fragment CTCAGgCACAAAggCgTA gT. S985 N fragment gCCATggTgCATggggTgACCA.

Sequence differences to the NCBI reference sequence (accession numbers NM\_005781.4) were identified via manual inspection of aligned chromatograms assisted by the Mutation Surveyor software package.

### 2.3. Plasmids construction

From pXJ-ACK1 WT, site-directed mutagenesis (SDM) was performed. Both forward and reverse primers comprising of the mutated residues were designed and SDM was performed using QuikChange<sup>®</sup> II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's instruction. Subsequently, ACK1 wild type and mutants were subcloned into BamHI and EcoRI site of pLXSN EKS (Clontech, Mountain View, CA).

### 2.4. Retrovirus production, infection and stable clone generation

2 × 10<sup>6</sup> Phoenix A cells was seeded in 6 cm plate 18 h prior to transfection. 8 µg pLXSN EKS plasmid DNA was transfected using Lipofectamine performed according to manufacturer's instructions (Invitrogen). 3 × 10<sup>5</sup> MCF7 cells/well were seeded in 6-well dish on the same day. 1.3 ml Fresh medium was added to the transfected Phoenix A cells medium the next day. 4 h Later, virus was harvested from the supernatant of the transfected Phoenix cells after filtering through 0.45 µm filter. 1 µl Of polybrene (8 mg/ml) was added into the filtered supernatant. The mixture was added to the MCF7 cells. Infection was repeated at 4 h interval for next 24 h. Infection efficiencies were monitored through the observation of control

GFP infected MCF7 cells using fluorescence microscopy. Stable clones were selected with 200  $\mu\text{g/ml}$  G418.

### 2.5. Silencing of ACK1 by siRNA duplexes

A498 cells were seeded into 24-well plates, incubated overnight and transfected at 30–40% confluency. ACK1-specific siRNA (Dharmacon RNAi Technologies, Lafayette, CO) or control scrambled siRNA were transfected by using Oligofectamine (Invitrogen) according to manufacturer's instructions. Cells were harvested for western blot after 72 h.

### 2.6. Silencing of ACK1 by shRNA

A498 cells were seeded into 6-well plates, incubated overnight, and transfected at 90% confluency followed by transfection of ACK1-specific shRNA plasmids (Origene, Rockville, MD), or control shRNA plasmids using Lipofectamine 2000<sup>®</sup> (Invitrogen) according to manufacturer's instructions. Medium was replaced with medium containing 0.5  $\mu\text{g/ml}$  puromycin for selection of stable cell line expressing the gene specific shRNA the next day.

### 2.7. Semi-quantitative PCR

RNA was isolated from cells using the RNeasy kit from Qiagen. 500 ng Total RNA was used to synthesis complementary DNA using Superscript III First-Strand Synthesis for RT-PCR (Invitrogen). 2  $\mu\text{l}$  Of the cDNA were loaded for Real-time PCR using ABI 7500 with SYBR green mix and respective primer sets design using Primer Express 3 (Applied Biosystems, Foster City, CA). ACK1 forward AgAgCCTgAAgACACgCACC reverse ggATCTgACTgCCgTTgAgg GAPDH forward CCCTTCATTgACCTCAACTACAT reverse TCCTggAAgATggTgATgg.

### 2.8. In vitro ubiquitination assay

$2.5 \times 10^6$  HEK293 cells were seeded in 10 cm plate. Cells were co-transfected with 7.5  $\mu\text{g}$  Myc-tagged ubiquitin and 10  $\mu\text{g}$  of FLAG-tagged ACK1 WT, mutants and MOP-1 (positive control) with Lipofectamine 2000<sup>®</sup> (Invitrogen) according to manufacturer's instructions. After 24 h, the cells were harvested and lysed with RIPA buffer. 500  $\mu\text{g}$  Lysate protein was immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG.

### 2.9. MG132 treatment

$4 \times 10^5$  HepG2 cells were seeded on each well of 6-well plate. 10  $\mu\text{M}$  MG132 were added to the cells and lysate were harvested at indicated time point for western blot.

### 2.10. Cycloheximide treatment

$4.5 \times 10^5$  cells/well MCF7 stable clones were seeded in 6-well plate the day before. 10  $\mu\text{g/ml}$  cycloheximide was added for 2, 4, 6, 8, 24 h. Cells were harvested at indicated times and lysed for immunoblotting. Signals were quantified using Image J and pixels at 0 h were set as 1. Protein half life was calculated using One phase decay method on Prism5. Half life ( $x$ ) =  $\ln(2)/K$  where K is the rate constant.

### 2.11. EGF stimulation

$4.5 \times 10^5$  cells/well MCF7 stable clones were seeded in 6-well plate. Serum-starved cells were treated with EGF (100 ng/ml) for the indicated times. The cells were harvested at different time points and the lysate was prepared for immunoblotting or immunoprecipitation.

### 2.12. Cell viability and apoptosis assay

2 Days post-siRNA-transfected cells were seeded in 96-well plate at 2500 cells/well. 24 h after seeding, cells were treated with 40  $\mu\text{M}$  of gefitinib for 8 h. Cell viability was assessed by CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) and Apoptosis was determined using Caspase-Glo 3/7 assay (Promega), according to manufacturer's instructions. Data are an average of two independent experiments with six replicates each.

### 2.13. Modified Boyden chamber assay

Serum-starved cells were trypsinised, counted and equal numbers were seeded into 8  $\mu\text{m}$  filter chamber (Corning Incorporated, NY). Medium containing 10% FCS was placed in the lower chamber, and cells were allowed to migrate for 6 h. Cells migrated to the underside were fixed and stained with 0.5% crystal violet. Experiments were performed in triplicate with five fields counted for each chamber and the average number of cells per chamber was calculated for each condition. Results are reported as averages of the three experiments (\*\* $p < 0.0001$ ).

### 2.14. Proliferation assay

Cells were trypsinised and equal numbers were seeded in 6-well plate accordingly at day 0. Subsequently, cells were trypsinised and counted using a particle counter (Beckman Coulter, Fullerton, CA) everyday for 4 or 6 days.

### 2.15. Scratch wound assay

$4.5 \times 10^5$  MCF7 stable clones were seeded in each well of 6-well plate and serum starved overnight. Scratch wound using 200  $\mu\text{l}$  tip was made across the well followed by two washes with PBS. 10% FBS were replaced in the medium and pictures were taken at 0 h and 20 h post-scratch.

### 2.16. Soft agar assay

7500 MCF7 stable clones were resuspended in 0.35% top agar and aliquoted on top of the 0.5% base agar in 6-well plate. The cells were left to form colonies in 37  $^{\circ}\text{C}$  incubation with 5% CO<sub>2</sub> for 21 days. Cells were fed two times a week. Colonies with more than 50 cells were counted under a dissection microscope after staining with 0.05% crystal violet. Data reported are average of three independent experiments (\*\* $p < 0.0001$ ).

### 2.17. Immunoprecipitation and western blot

Cells were scraped and lysed with pre-chilled RIPA buffer without SDS, and incubated on the ice for 30 min. The lysates were cleared by centrifugation at full speed for 10 min at 4 °C. Protein concentration was determined with standard BCA protein assay (Thermo Scientific, Rockford, IL). For immunoprecipitation (IP), 500 µg–1 mg of cell lysate was incubated with IP antibodies on ice for 30 min, followed by protein A + G beads (GE Healthcare, Giles, UK). The mixture was incubated at 4 °C overnight with rotation. The beads were washed with lysis buffer three times followed by the addition of SDS-PAGE sample buffer. The IP complexes were resolved in SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% milk in PBST (PBS with 0.1% tween). The primary antibodies used for immunoblotting include anti-phospho-Erk1/2, anti-phospho-Akt(ser473), anti-EGFR (Cell Signaling Technology, Beverly, MA) and anti-ACK1 (C20 and A11), anti-Myc, anti-Wwox (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PCNA, anti-EGFR, anti-phosphotyrosine 4G10 and anti-cyclin-D1 (Upstate, Lake Placid, NY); anti-β-actin-HRP (Abcam, Cambridge, UK); anti-E-cadherin, anti-N-cadherin, anti-phospho-p130<sup>cas</sup> and anti-fibronectin (BD Bioscience, Franklin Lakes, NJ); anti-FLAG M2, anti-HSP60 (Sigma). Secondary antibodies were anti-mouse and anti-rabbit horseradish peroxidase conjugated antibodies (GE Healthcare). Signals were detected with Super Signal West Dura Chemiluminescent substrate (Thermo scientific).

## 3. Results

### 3.1. Verification of somatic mutations – ACK1 S985 N and ACK1 A634 T in A498 and KatoIII, respectively

With the sequencing of transcripts from 90 tyrosine kinase genes in 261 cancer cell lines of diverse origins and 15 control tissues, we found 10 novel somatic- and two germ line mutations in the ACK1 gene (Fig. 1A) (Ruhe et al., 2007). Here, we verify two of the somatic mutations namely, ACK1 A634 T and ACK1 S985 N, in gastric (KatoIII) and kidney cancer cell line (A498), respectively. Sequencing of the genomic DNA and cDNA of A498 showed that a single nucleotide mutation in codon 985 (AGC–AAC) which resulted in a change from a serine to asparagine. This occurs only on a single allele (Fig. 1B, top). Subsequent sequencing of the cDNA showed that the mutant, but not the wild type allele, is expressed (Fig. 1B, bottom). Similarly for residue 634 mutation, a non-synonymous mutation (GCC–G/ACC) changed alanine to threonine on one allele. However, unlike the S985 N mutation, both wild type and mutant alleles were expressed in KatoIII cells (Fig. S1).

### 3.2. ACK1 S985 N mutation is kinase active leading to enhanced migration, proliferation and anchorage-independent growth

To characterize these mutations, we use a retroviral gene delivery system to create stable clones expressing ACK1 wild type (WT), Kinase dead (KD), S985 N, A634 T or GFP (control) in MCF7 cells that express low endogenous ACK1 protein.

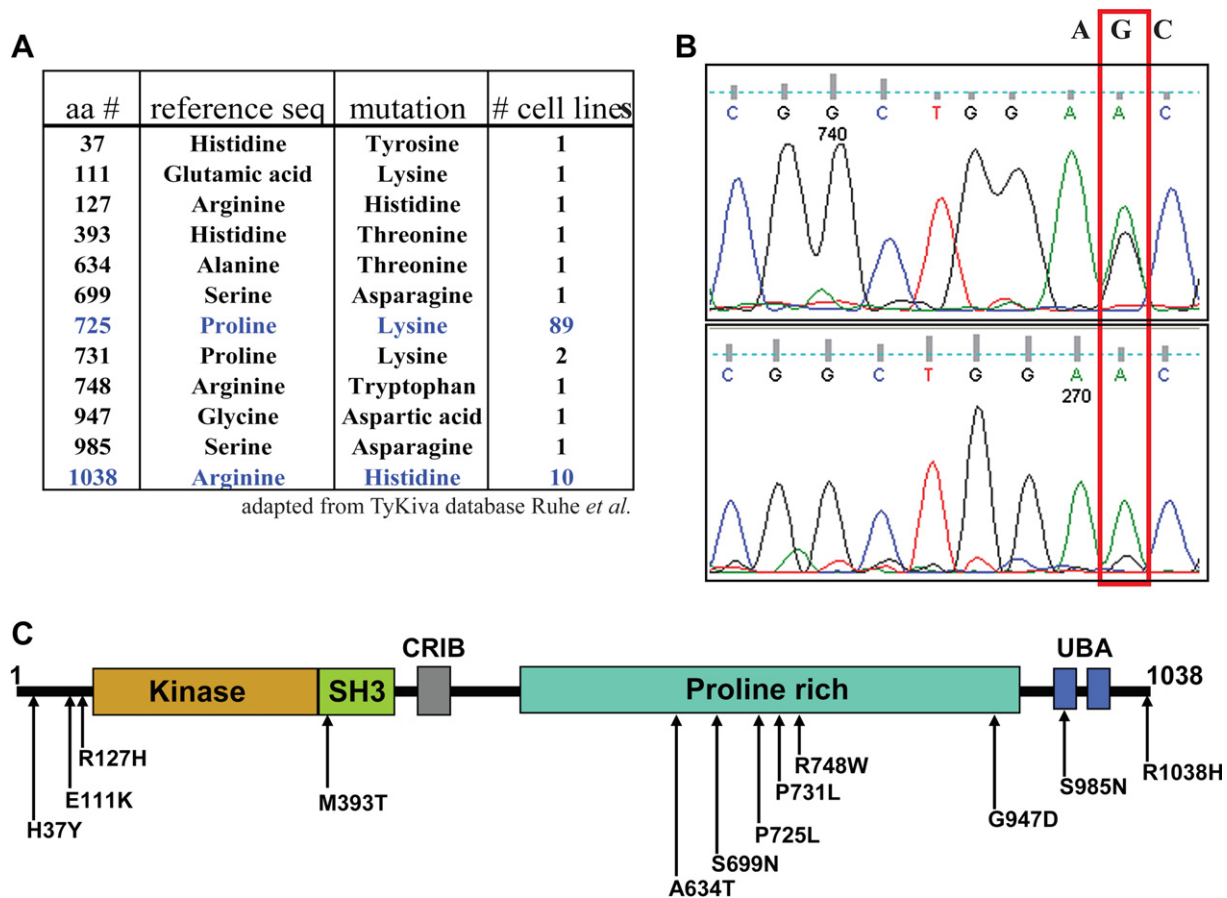
MCF7 cells were infected with retrovirus carrying constructs and stable clones were selected using 200 µg/ml G418. Both western blot (Fig. 2A) and immunofluorescence staining (data not shown) showed expression of ACK1 WT and mutant proteins, with exception of A634 T transfectants, which did not survive the selection. The overexpressed proteins were autophosphorylated as shown by immunoblotting with anti-phosphotyrosine antibody, 4G10 (Fig. 2B, upper panel). Additionally, western blotting of ACK1 substrate, p130<sup>cas</sup> revealed that the latter is phosphorylated thereby confirming that both WT and S985 N mutant ACK1 proteins retain their kinase activity (Fig. 2B, lower panel). In contrast, ACK1 KD protein could neither phosphorylate itself nor p130<sup>cas</sup>. The calf intestinal phosphatase (CIP) treated ACK1 WT lysate demonstrated the specificity of the phospho-p130<sup>cas</sup> antibody (Fig. 2B, lower panel) with β-actin as loading control.

Next, we studied the role of ACK1 S985 N mutation in cell migration using a modified Boyden chamber assay with 10% serum as chemoattractant. Stable expression of ACK1 WT in MCF7 cells increased migration vs GFP control (Fig. 2C, 55 vs 33%). MCF7 cells expressing ACK1 S985 N kinase showed a statistically significant 35% increase in chemotaxis compared to both GFP and ACK1 WT cells, suggesting that the point mutation at residue 985 of ACK1 enhances tumor cells migration. Increased motility conferred by ACK1 S985 N mutant was also supported by monolayer cell wound healing assay (Fig. 2D). Here, at 20 h post-scratch, it was evident that the ACK1 S985 N-expressing cells closed the wound more efficiently than ACK1 WT-expressing cells.

To investigate ACK1 S985 N's oncogenic potential, we checked both cellular proliferation by cell counting and anchorage-independent growth using the soft agar assay. For the proliferation assay, 50,000 cells were seeded in 6-well dish at day 0 and counted daily over a period of 4 days. Our data showed that at day 4, ACK1 S985 N stable cells have ~30% higher proliferation rate than WT-expressing cells, which in turn has ~40% higher proliferation rate than the GFP controls (Fig. 2EE). For the soft agar assay, 5000 cells were resuspended in top agar and aliquoted onto the base agar. Colonies were stained with crystal violet and counted after 21 days (Fig. 2F). Our data showed that MCF7 cells stably expressing ACK1 WT and S985 N are able to grow and proliferate in the soft agar compared to GFP-expressing cells. ACK1 S985 N expressing cells formed twofold more colonies which are also larger in size compared to those produced by the ACK1 WT.

### 3.2.1. Silencing of the ACK1 S985 N mutant protein in A498 cells reduced mitogenic signaling, motility, and proliferation

To validate the enhanced motility and proliferative role by ACK1 S985 N, we utilized a gene silencing strategy in the renal cancer cell line, A498, both transiently using siRNA and stably using shRNA. We successfully silenced ~70% ACK1 S985 N protein and mRNA expression in A498 cells using both siRNA and shRNA as demonstrated by immunoblotting (Fig. 3A, top panel) and real-time PCR (data not shown). Subsequent western blotting revealed strong reduction in Erk1/2 phosphorylation in ACK1-silenced A498 whereas reduced Akt phosphorylation was only observed in the stable ACK1 knock down cells (Fig. 3A).



**Figure 1** – Schematic diagram of ACK1 somatic- and germ line mutations identified in 261 cancer cell lines and 15 control tissues. (A) Summary of 10 somatic and two germ line (blue) mutations identified in ACK1 coding sequence (Ruhe *et al.*, 2007). (B) A498 genomic DNA (upper) and cDNA (lower) electropherogram showed single non-synonymous mutation at codon 985 – AGC–ACC. (C) Bar diagram shows the location of the identified mutations in the distinct domains on ACK1 protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We next looked at the consequences of ACK1 silencing on cell migration and proliferation. In contrast to the ACK1 S985 N overexpressing stable clone, our data demonstrated a ~50% reduction in migration between the shRNA control and ACK1-silenced A498 cells (50 vs 21 cells per wells) (Fig. 3B). In the proliferation assay with 30,000 A498 shRNA control and ACK1-silenced cells seeded on day 0, ACK1-silenced A498 cells showed four fold decreased proliferation at day 2 compared to the control (Fig. 3C). A reduction in proliferation rate was observed throughout the 6-day experiment.

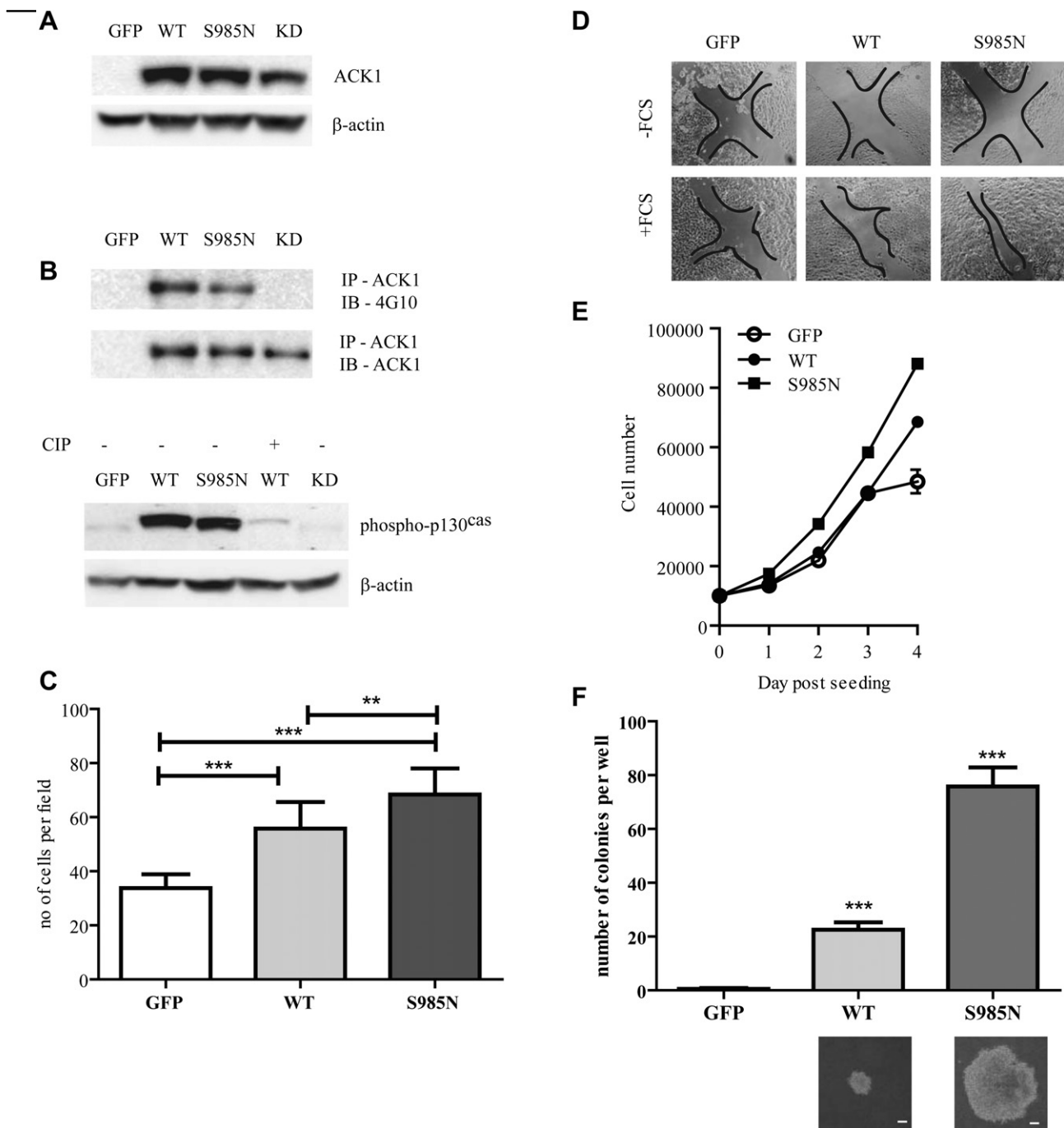
### 3.2.2. ACK1 plays a role in modulating EMT

It was reported by van der Horst *et al.* (2005) that ACK1 overexpression in HMEC resulted in enhanced cell motility and upregulation of fibronectin protein, an EMT marker. We next investigated the role of ACK1 S985 N in EMT by gene silencing. ACK1 knock down in A498 resulted in upregulation of the epithelial marker E-cadherin (more than 10-fold) and downregulation of mesenchymal markers. 72% and 50% Reduction of N-cadherin and fibronectin were observed, respectively, using shRNA (Fig. 3D). Since EMT is a process that requires changes in proteins and phenotype, the transient effect of siRNA is unable to clearly demonstrate the downregulation N-cadherin

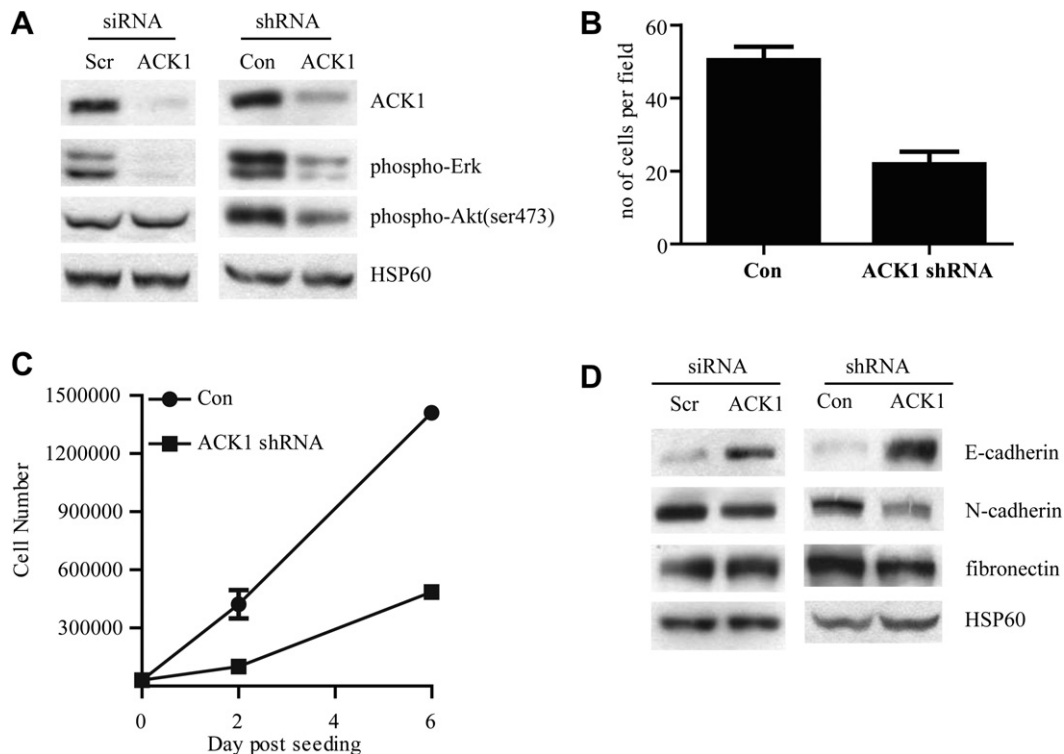
and fibronectin. Nonetheless, strong upregulation of E-cadherin and ~30% downregulation of N-cadherin were observed in the transient knock down cells. Consistent with this observation, decrease E-cadherin and increased N-cadherin were observed in the MCF7 stable cells expressing ACK1 S985 N (Fig. S2).

### 3.3. ACK1 S985 N mutant protein has lost its ubiquitin binding ability

Studies by others have shown that increased ACK1 activity in breast, androgen-independent prostate cancer correlates with tumor aggressiveness (Mahajan *et al.*, 2007; van der Horst *et al.*, 2005). Here, we attempted to gain an insight to the molecular mechanism where a single point mutation is capable of conferring these oncogenic phenotypes to A498. We first compared the ACK1 protein levels between A498 and a panel of cancer cell lines (Fig. 4A). Consistently, ACK1 protein levels appeared higher in aggressive cancer cell line such as MDA-MB 231 and A498 compared to MCF7 (Fig. 4A). This was, however, not due to gene amplification, as transcript levels (Fig. 4A) and gene copy numbers (data not shown) showed no difference with non-aggressive HEK293 or MCF7 cells.



**Figure 2 – ACK1 S985 N is kinase active leading to enhanced migration, proliferation and anchorage-independent growth.** MCF7 stable cell lysate expressing GFP, ACK1 WT, ACK1 S985 N and kinase dead (KD) protein were separated on 7% SDS-PAGE gel and immunoblotted with (A) anti-ACK1 and (B, bottom) anti-phospho-p130<sup>cas</sup> with anti- $\beta$ -actin as loading control. 1 U Calf alkaline phosphatase was incubated with the WT lysate for 30 min at 37 °C before SDS-PAGE. 500  $\mu$ g Lysate protein was immunoprecipitated with anti-ACK1 and immunoblotted with anti-phosphoTyr (4G10) or reprobred with anti-ACK1. (C) Serum-starved MCF7 stable cells were seeded in the upper side of the Transwell and 10% FCS was used as chemoattractant in the lower chamber. Cells were fixed and stained with 0.5% crystal violet blue after 6 h. Experiments were performed in triplicate with five fields counted for each chamber. Results are reported as averages of the three experiments. (D) Cells were grown to confluency and wounded (x) using a 200  $\mu$ l tip. Pictures were taken at 0 and 20 h post-scratch. (E) 10,000 MCF7 cells stably expressing GFP (○) ACK1 WT (●) and S985 N (■) were seeded on day 0. Subsequently, cells were trypsinised and counted using particle counter everyday for 4 days. Data reported as average of two independent experiments. (F) 5000 MCF7 stable cells were resuspended in 0.3% top agar and aliquoted onto 0.5% base agar. Cells were fed two times a week and incubated in 37 °C 5% CO<sub>2</sub> for 21 days. The colonies with more than 50 cells were counted under dissection microscope after staining with 0.05% crystal violet. Pictures of the colonies were taken under 10 $\times$  magnification. Scale bar represent 100  $\mu$ m. Data reported as average of three independent experiments. Statistical analysis is done using unpaired *t* test where \*\*\**p* < 0.0001.



**Figure 3 – Silencing of ACK1 S985 N in A498 cells reduced mitogenic signaling, motility, proliferation and EMT reversion.** (A) A498 cells were transfected with siRNA using Oligofectamine for 3 days or shRNA with Lipofectamine 2000 and selected with 0.5  $\mu\text{g/ml}$  puromycin. Cells were harvested and 50  $\mu\text{g}$  lysate proteins, control (Scr and Con) and ACK1-silenced A498, were immunoblotted with indicated antibodies. (B) Serum-starved control (Con) and ACK1-silenced A498 cells were seeded in the upper chamber of the Transwell and 10% FCS was used as chemoattractant in the lower chamber. Cells were fixed and stained with 0.5% crystal violet blue after 6 h. Experiments were performed in triplicate with five fields counted for each chamber. Results are reported as averages of the three experiments. (C) shRNA control (■) and silenced (●) A498 cells were seeded on day 0. Subsequently, cells were trypsinised and counted everyday for 6 days. Data reported as average of three independent experiments. (D) ACK1 was silenced using siRNA and shRNA as described in (A). 30  $\mu\text{g}$  Control (Scr and Con) and ACK1-silenced A498 cell lysate were immunoblotted with indicated antibodies.

Given the higher level of ACK1 in MDA-MB 231 and A498 cells, we next investigated if the protein level of ACK1 could be regulated by proteasomal degradation. Treatment with the proteasomal inhibitor MG132 of HepG2 that expresses low levels of ACK1 increased the endogenous level of ACK1 (Fig. 4B). A similar effect was observed on the positive control, Wwox, a tumor suppressor protein known to be regulated by proteasomal degradation (Mahajan et al., 2005).

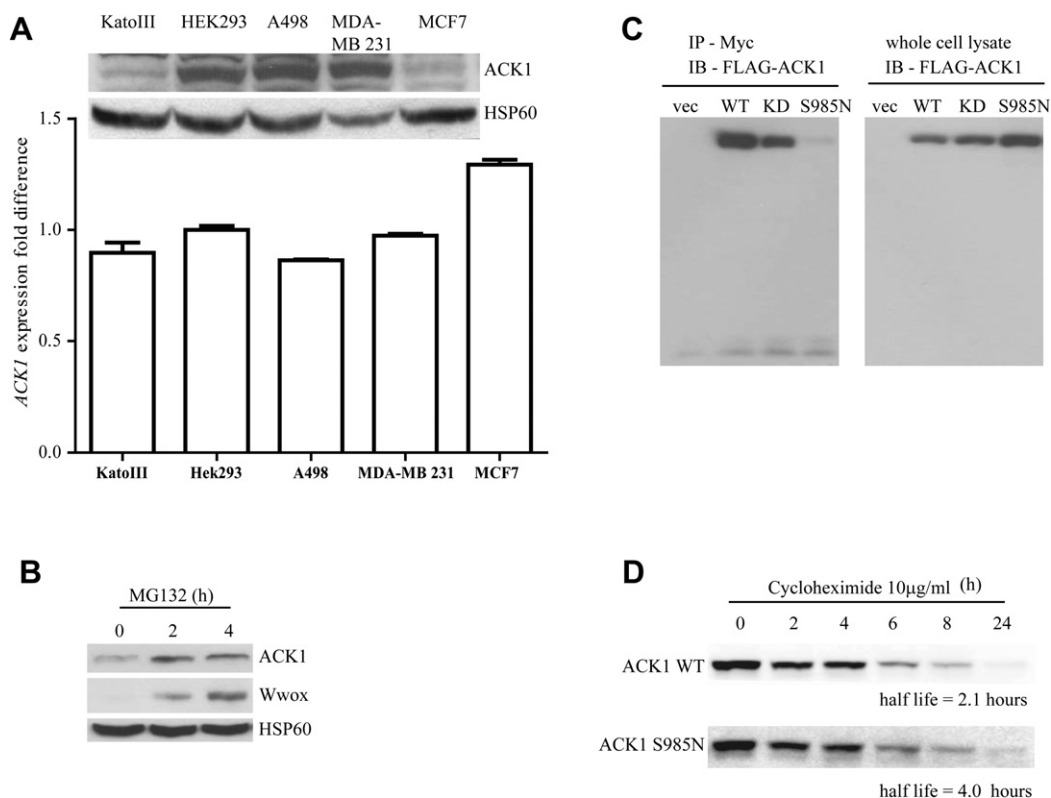
Since the ACK1 S985 N mutation is located on one of the two C-terminal UBA domains, we checked if this mutant ACK1 protein can be regulated by proteasomal degradation through ubiquitination. An *in vitro* ubiquitination assay with HEK293 co-transfected with Myc-tagged ubiquitin and FLAG-tagged ACK1 WT or ACK1 S985 N plasmids was set up (Fig. 4C). Ubiquitin conjugated proteins were immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-FLAG antibodies. Our results showed that only FLAG-tagged ACK1 WT but not ACK1 S985 N co-precipitated with Myc-tagged ubiquitin.

To confirm ACK1 S985 N resistance to ubiquitination and proteasomal degradation, we subjected the MCF7 stably expressing ACK1 WT and ACK1 S985 N to cycloheximide treatment (Fig. 4D). ACK1 S985 N which was unable to become

ubiquitinated displayed a longer protein half life compared to WT protein (4 and 2.1 h, respectively).

### 3.4. ACK1 S985 N is defective in EGFR downregulation

It had been established that ACK1 aids in the downregulation of EGFR receptor after EGF stimulation via the interaction with its MIG-6 like domain. This interaction targets the EGFR for ubiquitination and subsequent degradation (Shen et al., 2007), and requires the presence of the UBA domain on ACK1 (Shen et al., 2007). Since the S985 N mutant protein appeared to have lost its UBA binding function, we tested if EGFR can still be downregulated after stimulation. Serum-starved MCF7 stable clones expressing ACK WT or S985 N mutant were stimulated with EGF for specified times and the disappearance of total EGFR was observed by western blot. Our results showed that EGFR protein decreases within the first hour post-stimulation in ACK1 WT cells (Fig. 5A). This decrease was not observed for the ACK1 S985 N-expressing cells for up to 2 h and disappearance of EGFR protein was only detected after 4 h of stimulation. Longer EGFR signaling in ACK1 S985 N-expressing cells was supported by prolonged



**Figure 4 – ACK1 S985 N do not bind ubiquitin and has longer half life than ACK1 WT.** (A) 30 µg Of different cell lysates were separated on 7% SDS-PAGE and immunoblotted with anti-ACK1 and anti-HSP60 (upper panel). Real-time PCR was performed with ACK1-specific primers. The resulting average CT values were normalized against *GAPDH* mRNA as housekeeping control and data were expressed as a fold change in transcript expression vs expression in MDA-MB 231. Error bars represent standard deviation converted to fold changes ( $n = 3$ ). (B) HepG2 cells were seeded and treated with 10 µM MG132 for the indicated times. Cell lysate was harvested and immunoblotted with anti-ACK1, anti-Wwox and anti-HSP60 as loading control. (C) HEK293 cells were co-transfected with Myc-ubiquitin and FLAG-ACK1 WT, kinase dead (KD) and S985 N using Lipofectamine. 500 µg Cell lysate was immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG. 30 µg Whole cell lysate was loaded as expression control. (D) MCF7 stable clones expressing ACK1 WT and S985 N mutant were treated with 10 µg/ml cycloheximide for indicated time. Cell lysates were harvested and immunoblotted with anti-ACK1. Protein bands were scanned and intensity was quantified using Image J program and protein half life is calculated using One phase decay method (Prism5).

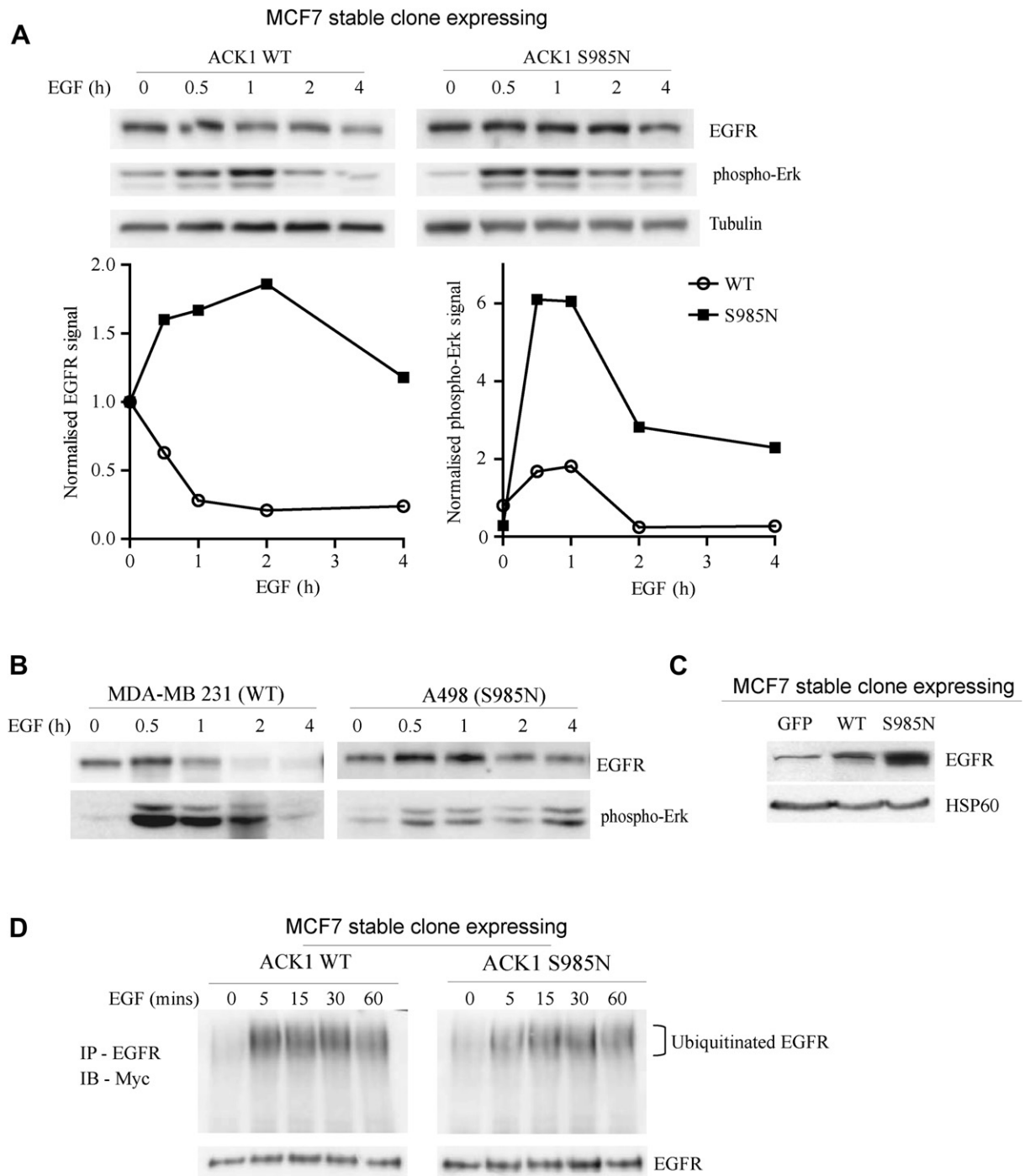
Erk phosphorylation and was further corroborated by using ACK1 WT-expressing MDA-MB 231 and A498 with ACK1 S985 N mutation (Fig. 5B). In addition, the deficiency in EGFR down-regulation was also reflected in the increase of the total EGFR level in the MCF7 cells stably expressing ACK1 S985 N (Fig. 5C).

Next we checked if the binding between ACK1 WT and S985 N mutant with EGFR could explain the deficiency in receptor downregulation. Both serum-starved MDA-MB 231 and A498 cells were stimulated with EGF. EGFR was immunoprecipitated from the whole cell lysate and western blot analysis was performed with the ACK1 antibody (Fig. S3). Our data showed that ACK1 WT protein co-immunoprecipitated with EGFR within 15 min of EGF stimulation and dissociated within 30 min. In contrast, interaction between ACK1 S985 N and EGFR remained detectable up to 30 min after EGF stimulation. From these observations, we next asked if ubiquitination of EGFR still occurs. In the MCF7 stable clones expressing ACK1 WT and ACK1 S985 N, we overexpressed Myc-tagged ubiquitin and EGFR WT. EGFR was immunoprecipitated and western blotted with anti-Myc antibodies. Here, it was seen that in ACK1 WT-expressing cells, receptor ubiquitination peaked

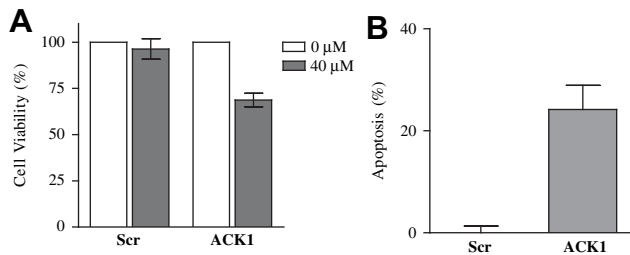
as early as 5 min post-EGF stimulation, whereas, in the ACK1 S985 N-expressing cells, EGFR ubiquitination was significantly delayed up to 30 min (Fig. 5D). This suggests that the ACK1 S985 N mutation resulted in extended binding with EGFR and delayed its ubiquitin-mediated degradation, thereby prolonging EGFR signaling.

### 3.5. Silencing ACK1 sensitized A498 to gefitinib treatment

Since ACK1 S985 N can play major role in both EMT and EGFR degradation, we next investigated if cell sensitivity to EGFR specific inhibitor was altered. Silencing of ACK1 was performed in A498 cells harboring the S985 N mutation. Subsequently, the cells were treated with gefitinib, an EGFR inhibitor commonly used in targeted therapy (Fig. 6). Interestingly, although the EGFR protein level was high in A498 cells, it is resistant to 8 h of 40 µM gefitinib treatment. However, gefitinib-induced toxicity was observed in ACK1-silenced A498 with a 25% reduced cell viability (Fig. 6A) and a corresponding increase in caspase activation



**Figure 5 – ACK1 S985 N maintains EGFR level after stimulation through reduced receptor ubiquitination. (A)** Serum-starved MCF7 stable clones expressing ACK1 WT or S985 N mutant were stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were harvested and immunoblotted with the indicated antibodies. Signals were normalized to the loading control, using Image J software. **(B)** Serum-starved MDA-MD 231 and A498 were stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were harvested and immunoblotted with anti-EGFR and anti-phospho-Erk. **(C)** Cell lysate of MCF7 stable clones expressing ACK1 WT or S985 N were harvested and immunoblotted with anti-EGFR and anti-HSP60. **(D)** MCF7 stable clones were transfected with Myc-ubiquitin and pcDNA-EGFR construct with Lipofectamine 2000. Cells were starved and stimulated with 100 ng/ml EGF for the indicated times. 500 µg Of lysate protein were immunoprecipitated with anti-EGFR and immunoblotted with anti-Myc or reprobred with anti-EGFR.



**Figure 6 – Silencing ACK1 sensitized renal carcinoma cells to gefitinib.** A498 were transfected with scrambled (scr) or ACK1 siRNA for 2 days. Cells were re-seeded in 96 wells plate and treated with 40  $\mu$ M gefitinib for 8 h. (A) Cells viability was measured by CellTiter Glo and results expressed as a percentage of viable cells to the untreated controls. (B) Apoptosis was measured using Caspase3/7 assay and result is expressed as percentage of apoptosis to the untreated cells. Both assays were done in two independent experiments with six replicates each.

(Fig. 6B). These data strongly suggest that the oncogenic role of ACK1 S985 N mutation supports resistance to an anti-cancer therapeutic.

#### 4. Discussion

The broad importance of PTKs in tumorigenesis has been demonstrated over many years. Oncogenic mutations have been identified in clinical samples as well as cancer cell lines [reviewed in (Zhang et al., 2009)]. In our work, we addressed the question if an oncogenic role is conferred on ACK1 by a change in its stability. We characterized two missense mutations which resulted in a single amino acid change at the E3 ligase binding domain and UBA in gastric and renal cancer cells, respectively. In this study, the effect of the somatic mutation was analyzed using MCF7 stable clone overexpression and gene silencing in A498 cells. We demonstrated that the ACK1 S985 N mutation enhances cell proliferation, migration, anchorage-independent growth as well as mitogenic signaling, compared to the wild type kinase. Interestingly, silencing of ACK1 (S985 N) in A498 cells resulted in an upregulation of epithelial markers suggestive of EMT reversion. Our results also demonstrated that ACK1 with a mutation at residue 985 loses its ability to bind ubiquitin, which stabilized the kinase and impaired its role in EGFR downregulation and degradation through receptor ubiquitination and proteasomal degradation.

ACK1 has been clearly implicated in cancer progression in recent years. A strong correlation between ACK1 gene copy number, protein level and activity have been demonstrated in tumors of different tissue types (Mahajan et al., 2007; van der Horst et al., 2005; Wang et al., 2006). Our western blot analysis of different cancer cell lines confirmed a correlation of ACK1 protein levels with cancer aggressiveness. ACK1's role in proliferation and migration are clearly demonstrated and supported by reduced MAPK signaling in ACK1-silenced cells. Evidently, transient silencing of ACK1 does not affect Akt phosphorylation suggesting that ACK1 does not influence cellular metabolism and survival which are largely controlled by Akt. However, we do observed a reduction in Akt

phosphorylation after prolonged ACK1 silencing using shRNA. This is probably due to a feedback mechanism as well as cross talk between the MAPK and Akt pathway in cells.

In our work, in addition to the ACK1 WT potential in tumorigenicity, we also identified an enhanced migratory phenotype of the ACK1 S985 N mutant and its significant role in EMT in the A498 kidney carcinoma cells. The latter result is consistent with the observations of van der Horst et al. (2005) in which ACK1 WT was overexpressed in HMEC cells. Unlike their model where only fibronectin was upregulated, we showed here that in ACK1-silenced A498 cells, there was a strong upregulation of E-cadherin, an epithelial marker and downregulation of both N-cadherin as well as fibronectin. These differences suggest that the enhanced oncogenic ability arose from the S985 N mutation of ACK1 in A498 cells. The data convincingly implicated the involvement of ACK 1 in EMT as well as the role of ACK1 S985 N in A498 renal cancer cells. Its has been shown recently that A498 conditioned media could enhance invasiveness of 786-O, a less aggressive form of renal cell line (Chuang et al., 2008). The authors had identified involvement of several cytokines including TNF- $\alpha$ , Interleukin-1 $\beta$ , Interleukin-6, hypoxia-inducible factor- $\alpha$  and matrix metalloproteinase-2, of which TNF- $\alpha$  plays an important role. It would be exciting to further investigate how ACK1 contributes to EMT and if this involves the regulation of TNF- $\alpha$ .

In addition to EMT, our finding also demonstrated that the single amino acid mutation at residue 985 contributed to ACK1 protein stability with enhanced EGFR binding but impaired receptor ubiquitination and downregulation. These data are well supported by others showing the importance of the UBA domain on ACK1 for EGFR downregulation (Shen et al., 2007) and especially a recent publication by Chan et al. (2009), who demonstrated that overexpression of ACK1 blocked EGFR ubiquitination. This inhibition is overcome by co-expression of the Nedd4-2 E3 ligase which rapidly ubiquitinates and degrades ACK1. Here, we showed that the mutation on residue 985 on ACK1, resulted in a loss of its ubiquitin binding property. Thus, ACK1 S985 N does not respond to E3 ligase degradation and remain bound to EGFR in the presence of EGF stimulation. The physical binding of the ACK1 mutant might serve to reduce the ability of c-cbl to ubiquitinate EGFR, resulting in continuous mitogenic signaling in the renal cancer cells. The intriguing question is whether the level of Nedd4-2 E3 ligase also plays an important role in cancer development. Further, given that the EGFR-ACK1 S985 N protein complex dissociates after prolonged EGF stimulation, is there a proteasome-independent protein degradation pathway at play that removed ACK1 from the EGFR? All these questions await further investigation. Nonetheless, our data provide an explanation on how cancer cells could gain their oncogenic phenotype through various mutations of ACK1.

In view of the effects of S985 N on ACK1 stability, we also attempted to characterize the second mutation found in gastric cancer cell line. The A634 T mutation located on the Nedd4-2 E3 ligase binding domain was verified in KatolIII. Unlike A498 cells, ACK1 of both wild type and mutant proteins are translated. An *in vitro* ubiquitination assay demonstrated that, like S985 N, the A634 T mutant is unable to bind ubiquitin (data not shown), suggesting that this mutation in the E3 ligase binding domain could confer stability to the kinase and

therefore might be deficient in EGFR downregulation. However, we could not further investigate its role in EGFR regulation as we could not obtain a viable stable clone expressing A634 T constructs after G418 selection. Based on our data on ACK1 S985 N, it is highly likely that A634 T would behave similarly.

Renal cell carcinoma (RCC) accounts for more than 12,000 cancer deaths in the United States (Jemal et al., 2003). The current treatment includes surgical resection followed by interleukin-2 or Interferon- $\alpha$  treatment. Some patients experience distant metastatic lesions of RCC. In the last 5 years, receptor tyrosine kinases have become prominent anti-tumor targets for cancer therapy development including RCC. Currently, two tyrosine kinase inhibitors namely, sunitinib and sorafenib are approved by FDA for RCC treatment. In addition, there are several clinical trials of different phases for RCC, of which, EGFR, VEGFR and PDGFR inhibitors are being investigated for their potency (Potti and George, 2004). Interestingly, high EGFR protein in RCC has been reported since 1989 (Sargent et al., 1989; Uhlman et al., 1995), however, it is not clear if this is due to overexpression of the receptor or defective receptor downregulation. In this work, we propose that the high EGFR found in A498 RCC is due to ACK1 S985 N that prevents receptor downregulation, resulting in constitutively strong mitogenic signaling in the cells. In addition, despite high EGFR protein level, A498 remained highly resistant to gefitinib treatment. Our mechanistic studies on ACK1 in A498 suggested a regulatory role in EGFR downregulation. Indeed, silencing of ACK1 sensitized cancer cells to gefitinib. A high gefitinib concentration was used in our experiment to accommodate the transient siRNA silencing effect. We expect similar, if not better sensitization, at repeated low dosage treatment of the ACK1-silenced cells. Taken together, these findings open up an opportunity of combinational therapy against ACK1 not only in high EGFR expressing renal tumors, but also in lung cancer where a large percentage of tumors overexpressing EGFR are also resistant to EGFR inhibitors.

## 5. Conclusion

We characterized a novel point mutation, S985 N on ACK1's UBA domain. This mutation renders the ACK1's role on EGFR downregulation inefficient which resulted in constitutively high mitogenic signal transduction in the tumor cells. Although the work focuses on ACK1 extended stability and its effect on EGFR degradation, nonetheless, it also provides a probable explanation on how the high ACK1 protein observed in cancer cells could promote tumorigenesis. Currently, we are in the process of collecting and sequencing ACK1 in clinical tumor samples from various tissue types. It would be very exciting to see if we could locate similar mutations in different tumor types that have been associated with high ACK1 expression levels.

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## Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molonc.2010.03.001.

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