ORIGINAL ARTICLE

Knockout of c-Cbl slows EGFR endocytic trafficking and enhances EGFR signaling despite incompletely blocking receptor ubiquitylation

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Abstract

Epidermal growth factor receptor (EGFR) activity is necessary and sufficient for corneal epithelial homeostasis. However, the addition of exogenous Epidermal Growth Factor (EGF) does not reliably restore the corneal epithelium when wounded. This is likely due to high levels of endogenous EGF in tear fluid as well as desensitization of the EGFR following ligand stimulation. We hypothesize that preventing receptor downregulation is an alternative mechanism to enhance EGFR signaling and promote the restoration of compromised corneas. Ligand-dependent EGFR ubiquitylation is associated with the targeted degradation of the receptor. In this manuscript, we determine whether knockout of c-Cbl, an E3 ubiquitin ligase that ubiquitylates the EGFR, is sufficient to prolong EGFR phosphorylation and sustain signaling. Using CRISPR/Cas9 gene editing, we generated immortalized human corneal epithelial (hTCEpi) cells lacking c-Cbl. Knockout (KO) cells expressed the other E3 ligases at the same levels as the control cells, indicating other E3 ligases were not up-regulated. As compared to the control cells, EGF-stimulated EGFR ubiquitylation was reduced in KO cells, but not completely abolished. Similarly, EGF:EGFR trafficking was slowed, with a 35% decrease in the rate of endocytosis and a twofold increase in the receptor half-life. This resulted in a twofold increase in the magnitude of EGFR phosphorylation, with no change in duration. Conversely, Mitogen Activating Protein Kinase (MAPK) phosphorylation did not increase in magnitude but was sustained for 2–3 h as compared to control cells. We propose antagonizing c-Cbl will partially alter receptor ubiquitylation and endocytic trafficking but this is sufficient to enhance downstream signaling.

Significance Statement

Ligand-mediated ubiquitylation is a key component of EGFR downregulation; preventing EGFR ubiquitylation is a novel strategy for restoring homeostasis to the corneal epithelium. Knockout of c-Cbl in human corneal epithelial cells decreases but does not

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completely abrogate ligand-mediated receptor ubiquitylation, but the reduced EGFR ubiquitylation is sufficient to slow EGFR degradation and sustain receptor phosphorylation and downstream signaling. Antagonizing c-Cbl may be a novel pharmacological strategy for the restoration of damaged corneal epithelium.

KEYWORDS
c-Cbl, corneal epithelium, EGFR, membrane trafficking, ubiquitylation

1 | INTRODUCTION

The Epidermal Growth Factor Receptor (EGFR) is a nearly ubiquitously expressed receptor tyrosine kinase that has biological roles in zygote implantation, tissue development, and tissue homeostasis. Overexpression and hyperactivation of the EGFR is a characteristic of many cancers and is associated with poor prognosis.[1]

One tissue the EGFR regulates is the corneal epithelium. The cornea is the anterior portion of the eye and is the first anatomical barrier to the environment. There are three cell layers of the cornea—the epithelium, stroma, and endothelium—that are separated by the Bowman's and Descemet's layers, respectively.[2] The outermost epithelial layer is highly innervated, and perturbation of this layer is very painful. A fully differentiated corneal epithelium is critical to properly refract light onto the retina and to keep foreign substances (i.e., bacteria, viruses, small particles) out of the immune-privileged eye.

EGFR signaling is both necessary and sufficient for homeostasis and regeneration of the corneal epithelium. Patients taking cetuximab, an EGFR inhibitor that prevents cancer progression, have increased incidents of corneal erosions.[3–5] In the laboratory, adding EGF to corneal wounds in rodent and rabbit models accelerates their healing.[6] However, in the clinics, the addition of exogenous EGF does not reliably accelerate corneal epithelial wound healing. In one study, patients with corneal abrasions and epithelial lesions saw improved healing with EGF treatment,[7] as did corneal erosions associated with cetuximab treatment[8,9] and traumatic corneal ulcers.[10] In contrast, topical EGF does not help patients with herpes simplex dendritic ulcers, bullous keratopathies, stromal keratitis, or penetrating keratoplasty.[7,11,12]

One reason that corneal epithelial perturbations might be refractory to exogenous EGF is the high levels of EGF in tears. Tear fluid from healthy volunteers with no apparent ocular pathology have EGF levels of ~2 ng/ml (0.32 nM).[13–16] This concentration is close to the Kd of EGF for the EGFR. Thus, under basal conditions, there is substantial receptor occupancy and additional EGF will not appreciably increase EGFR signaling. Further, constant stimulation of the EGFR with exogenous EGF will lead to the lysosomal degradation of the ligand:receptor complex and attenuated signaling.[17]

In this study, we explored alternative approaches to enhance EGFR signaling. Specifically, we sought to inhibit the ligand-mediated ubiquitylation of the EGFR, a post-translational modification that targets the receptor for lysosomal degradation. We hypothesized that the inhibition of receptor ubiquitylation would be sufficient to slow receptor degradation and sustain signaling.

Ubiquitylation is a multienzyme process activated by a high-energy thioester linkage between the ubiquitin and an E1 (ubiquitin-activating enzyme) protein.[18] The ubiquitin is transferred to a Ubiquitin-conjugating (UBC) enzyme, E2, forming a second thioester linkage. The RING finger of an E3 protein binds with the target substrate and a ubiquitin-charged E2 and facilitates the transfer of ubiquitin from the E2 to the substrate. Ultimately, this results in the covalent attachment of ubiquitin to the substrate.[19] The ubiquitylated EGFR is targeted for lysosomal degradation.[20,21] However, it is not clear if ubiquitylation regulates receptor endocytosis,[22,23] lysosomal degradation,[24,25] or both facets of membrane trafficking.

All members of the Cbl family (c-Cbl, Cbl-b, and Cbl-3) have been reported to be E3 ligases for the EGFR,[19,26,27] as well as NEDD4.[28,29] However, most studies have used cancer cells[27] or cell lines with exogenously expressed EGFRs.[24,30] There is little information regarding which E3 ligase mediates ubiquitylation and how ubiquitylation regulates endogenous EGFR function in the corneal epithelium. This foundational information is critical in the design of pharmacological agents to promote corneal epithelial homeostasis.

We used CRISPR/CAS9 technology to make clonal isolates of c-Cbl knockout immortalized human corneal epithelial (hTCEpi) cells.[31] The absence of c-Cbl did not affect the expression of other E3 ubiquitin ligases. The loss of c-Cbl did not completely prevent ligand-mediated EGFR ubiquitylation, but it did reduce both the rate of ligand:receptor endocytosis and intracellular endocytic trafficking. Functionally, c-Cbl knockout cells had increased EGFR and MAPK signaling. Together these data indicate that in corneal epithelial cells, c-Cbl is a primary mediator of EGFR ubiquitylation. The loss of c-Cbl and EGFR ubiquitylation ultimately promotes EGFR activity.

2 | MATERIALS AND METHODS

2.1 | Cells

hTCEpi cells were obtained from Evercyte. Human corneal epithelial cells were immortalized by the stable transfection of human telomerase reverse transcriptase.[31] Cells were grown in Keratinocyte Basal Medium (KBM) with growth supplement (Lonza,) at 37°C and were maintained at 5% CO_2.

2.2 | CRISPR/CAS9

Cells were transduced with a lentivirus encoding CAS9 and blasticidin resistance gene. Blasticidin resistant colonies were isolated and transfected with annealed tracrRNA (mA*mG*CAUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGG UGCU*mU*mU) and c-Cbl guideRNA (crRNA Sequence: mC*mA*UC UUUACCCGACUCUUUCGUUUUAGAGCUAUG*mC*mU and crRNA Sequence: mC*mU*AUUCUUUAGCGCCAGCUUGUUUUAGAGCU AUG*mC*mU). Cells were plated at low density and individual colonies were isolated, amplified, and screened by immunoblot. In total, 27 colonies were screened with three positive clones. Experiments were performed on multiple clones with indistinguishable results.

2.3 | Antibodies

Antibodies were obtained from the following sources: EGFR (A-10, Santa Cruz Biotechnology), EGFR (Ab-1, EMD Millipore), EEA1 (#C45B10, Cell Signaling); pY1068 (#3777, Cell Signaling), Ubiquitin (SC-8017, Santa Cruz Biotechnology), α-tubulin (Sigma-Aldrich), c-Cbl (#2747, Cell Signaling); Cbl-B (#9498, Cell Signaling), NEDD4 (#2740, Cell Signaling), Cbl-3 (#A305-043A-M, Thermo Fisher Scientific).

2.4 | Cell treatment

Before experimentation, cells were serum starved by washing twice with PBS pH 7.4 and incubating in serum-free media for 2 h. Cells were treated with the indicated concentrations of EGF for the indicated periods of time.

2.5 | Cell lysate preparation and immunoblotting

EGF-treated hTCEpi cells were washed twice with room temperature PBS pH 7.4, followed by the addition of PBS pH 7.4, and equilibration to 4°C on ice. The PBS was removed and cells were harvested in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 10 mM sodium pyrophosphate, 100 mM sodium fluoride) supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF), solubilized with end over end rotation for 10 min at 4°C. Insoluble material was removed by centrifugation for 10 min at 4°C and maximum speed (21,130 rcf) in an Eppendorf 5424R. Equivalent amounts of cell lysate (indicated in the figure legends) were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were immunoblotted with the indicated primary antibodies according to the manufacturer's recommendation. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized using enhanced chemiluminescence and a Fotodyne Imaging system.

2.6 | EGFR ubiquitylation

Epidermal growth factor receptor ubiquitylation was monitored using a modification of a protocol by Visser Smit et al.[32] Serum-starved cells were treated with 50 ng/ml of EGF in Keratinocyte Serum-Free Media (K-SFM) with no additions for 0, 2, or 10 min, and harvested in 4°C EGFR-UB lysis buffer (0.5% Triton x-100/50 mM Tris pH 7.5/150 mM NaCl/1 mM EDTA/1 mM sodium orthovanadate/10 mM sodium fluoride) supplemented with 2 mM PMSF (Calbiochem)/16 μM G5 Ubiquitin isopeptidase inhibitor I (Santa Cruz Biotechnology). Cell lysates were prepared and immunoprecipitated with 1 μg EGFR antibody (mouse monoclonal, clone 528), (Ab-1, EMD Millipore), incubated at 4°C overnight followed by another 2 h incubation at 4°C with protein A/G Agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed thrice in chilled EGFR-UB lysis buffer. Proteins were eluted with 6XSDS sample buffer and separated by 7.5% SDS-PAGE and immunoblotted for EGFR, Ub, or pY1068 EGFR as indicated. Immunoblots were quantified using NIH ImageJ software, taking care to make sure the exposures were in the linear range.

2.7 | Texas Red-EGF labeling of cells

Cells were incubated with 2 μg/ml of Texas Red-EGF (Invitrogen) for 10 min at 37°C and washed twice with cold modified PBS (PBS pH7.4, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, 0.2%BSA), three times with cold citrate buffer pH 4.6 (25.5 mM citric acid, 24.5 mM sodium citrate, 280 mM sucrose), and re-equilibrated with two PBS washes. Cells were returned to 37°C serum-free media. At the appropriate time point, cells were fixed in 4% paraformaldehyde and processed for indirect immunofluorescence as described below.

2.8 | Immunofluorescence

Treated cells were fixed in 4% p-formaldehyde/PBS++ (PBS/ 0.5 mM $MgCl_2$/0.5 mM $CaCl_2$) solution at room temperature for 5 min and on ice for 15 min. Excess formaldehyde was removed with three 5-min washes in PBS++. Cells were then permeabilized for 20 min in 0.1% saponin/5% FBS/PBS++ and washed three times with PBS++ (5 min each). After washing, cells were incubated for 1 h at room temperature with the EGFR mouse monoclonal antibody (clone 528), Ab-1 (Millipore Sigma). Unbound primary antibody was removed with 3 × 5 min washes in PBS++ and cells were incubated for 1 h with an Alexa488 conjugated goat anti-mouse secondary antibody (Molecular Probes). After six 10 min washes with PBS++, coverslips were rinsed in Millipore water and mounted on a slide with Prolong Antifade with DAPI (Thermo Fisher Scientific). Images were collected Nikon Eclipse Ti-E microscope using Nikon NIS Elements software. The images were then exported to Adobe Photoshop for the preparation of figures.

2.9 | ^{125}I-EGF internalization/degradation

Radioligand trafficking was performed as described by Vanlandingham and Ceresa.[33] For internalization assays, 35 mm dishes of hTCEpi cells were incubated with 1 ng/ml of ^{125}I-EGF (catalog number NEX160; PerkinElmer Life Sciences; specific activity 150–200 µCi/µg) for 2 h on ice to achieve steady-state binding. Following the removal of unbound radioligand with four washes of ice-cold binding buffer, cells were incubated with 37°C binding buffer and incubated at 37°C. At the indicated times, cells were removed from the incubator. Media was collected. Cell surface ^{125}I-EGF was collected by pooling two 8-min washes of the cells with 0.5 M acetic acid/0.5 M NaCl. Internalized ^{125}I-EGF was collected by solubilizing the remaining cells with 0.1 M NaOH/0.1% SDS. Each fraction was counted in a Wizard gamma counter (PerkinElmer). Rate constants for internalization were calculated as described by Jiang et al.[34]

For intracellular trafficking assays, 35 mm dishes of hTCEpi cells were incubated with 1 ng/ml of ^{125}I-EGF for 7 min at 37°C to internalize the radioligand. Cells were removed from the incubator, put on ice, and washed four times with ice-cold binding buffer. Cells were returned to 37°C. At the indicated times, cells were removed, and the media was collected to measure secreted radioligand.[35] The remaining cells were collected in 1% Nonidet P-40, 20 mM Tris, pH 7.4, and precipitated on ice for 1 h using 10% trichloroacetic acid (TCA) and 0.1% bovine serum albumin (BSA) as a carrier. Intact (pellet) and degraded (supernatant) fractions were separated by 15 min of centrifugation at 4°C. Each fraction was counted in a Wizard gamma counter (PerkinElmer). The secreted fraction was calculated as the radioactivity in the media/total radioactivity. The percent of intact radioligand was calculated as the amount of intact radioactivity/total intracellular radioactivity.

3 | RESULTS

3.1 | Knockdown of c-Cbl in corneal epithelial cells does not block EGFR ubiquitylation

The E3 ligase c-Cbl was knocked out of human telomerase reverse transcriptase immortalized human corneal epithelial (hTCEpi) cells.[31] Cells were first transduced with a retrovirus encoding CAS9 and the blasticidin resistance gene; blasticidin resistant cells were transfected with gRNA targeting human c-Cbl. Individual clones were isolated, amplified, and screened for the loss of c-Cbl expression. Three of the 27 clones amplified were negative for c-Cbl expression as determined by immunoblot. In contrast, there is no change in Cbl-b, Cbl-3, or NEDD4 expression as compared to the CAS9 expressing cells (Figure 1A).

To determine if the knockout of c-Cbl was sufficient to block ligand-mediated EGFR ubiquitylation, parental and knockout cells were treated with 50 ng/ml of EGF for varying amounts of time (0–10 min). Cell lysates were prepared and immunoprecipitated for the EGFR. The resulting immunoprecipitates were divided into thirds, and immunoblotted for Ubiquitin (Ub), phosphorylated EGFR (pY1068), and total EGFR (Figure 1B) to assess receptor ubiquitylation, stimulation, and as a immunoprecipitation control, respectively. Knockout of c-Cbl reduced the amount of ligand-dependent EGFR ubiquitylation,
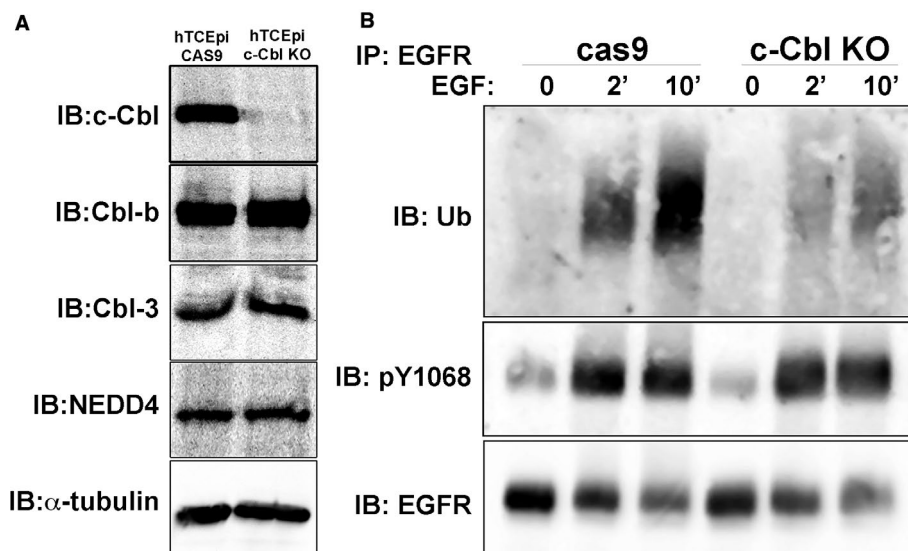

FIGURE 1 C-Cbl knockout in hTCEpi cells reduces ligand-mediated EGFR ubiquitylation. c-Cbl was knocked out of hTCEpi cells using CRISPR/CAS9 technology (see Materials and Methods). (A) Cell lysates were prepared from hTCEpi cells expressing either only CAS9, or CAS9 and a human c-Cbl specific gRNA. Equivalent amounts of cell lysate (20 µg) were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for c-Cbl, Cbl-b, Cbl-3, Nedd4, or α-tubulin. Shown is a representative blot from at least three experiments. Indistinguishable results were observed with other clones. (B) CAS9 or c-Cbl knockout cells were serum-starved and then treated with 50 ng/ml of EGF for 0, 2, 10 min. Cell lysates were prepared, and the EGFR was immunoprecipitated (Ab-1, EMD Millipore). Immunoprecipitates were divided into thirds, resolved by 7.5% SDS-PAGE, and immunoblotted using antibodies against ubiquitin (Ub), phosphorylated EGFR (pY1068), and EGFR. Shown is a representative experiment repeated at least three times with multiple clones

but did not completely eliminate it. Over the course of 10 min of EGF treatment, the levels of total and phosphorylated EGFR in c-Cbl knockout cells were indistinguishable from the control cells.

3.2 | Loss of c-Cbl does not block EGFR trafficking but alters the rate and route

To assess how the reduction in EGFR ubiquitylation affected the trafficking of the EGF:EGFR complex, we monitored the localization of fluorescently labeled EGF (Texas Red-EGF) in cells with (CAS9 cells) and without c-Cbl (-c-Cbl) (Figure 2). Briefly, cells were incubated with Texas Red-EGF that was allowed to internalize for 10 min at 37°C. Free and cell surface-bound ligand was removed. The intracellular Texas Red-EGF was allowed to traffic through the cell for the indicated amounts of time (0–60 min) and stained by indirect immunofluorescence for the early endosome auto-antigen 1 (EEA1), a marker of the early endosome.[36] In the absence of c-Cbl, Texas Red-EGF was able to internalize and traffic within the cell with no significant changes in the distribution of the fluorescent ligand.

These studies were complemented with indirect immunofluorescence experiments that monitor ligand-stimulated EGFR internalization (Figure 3). In the CAS9 cells, EGF treatment causes the receptor to redistribute into the cytoplasm resulting in very little plasma membrane EGFR staining at the 30 and 60 min time points. Although the majority of EGFRs were able to internalize in c-Cbl knockout cells, there is a more pronounced EGFR staining at the plasma membrane 15–60 min after ligand treatment.

Next, we used ^{125}I-EGF to quantify ligand endocytosis, degradation, and secretion (Figure 4). We found that the knockdown of c-Cbl is associated with a 35% decrease in the rate of EGFR endocytosis (Figure 4A and B). The amount of ^{125}I secreted was increased at 30–90 min in c-Cbl KO cells (Figure 4C).[35] Interestingly, the c-Cbl knockout cells had a rate of intracellular ^{125}I-EGF degradation that was indistinguishable from the CAS9 cells at every time point except 2 h (Figure 4D).

3.3 | The loss of c-Cbl enhances EGF-mediated receptor signaling

To assess how these changes in membrane trafficking impacted the signaling of the EGFR, we treated control and c-Cbl knockout cells with EGF for varying amounts of time (0–240 min) and monitored the kinetics of EGFR degradation and phosphorylation/
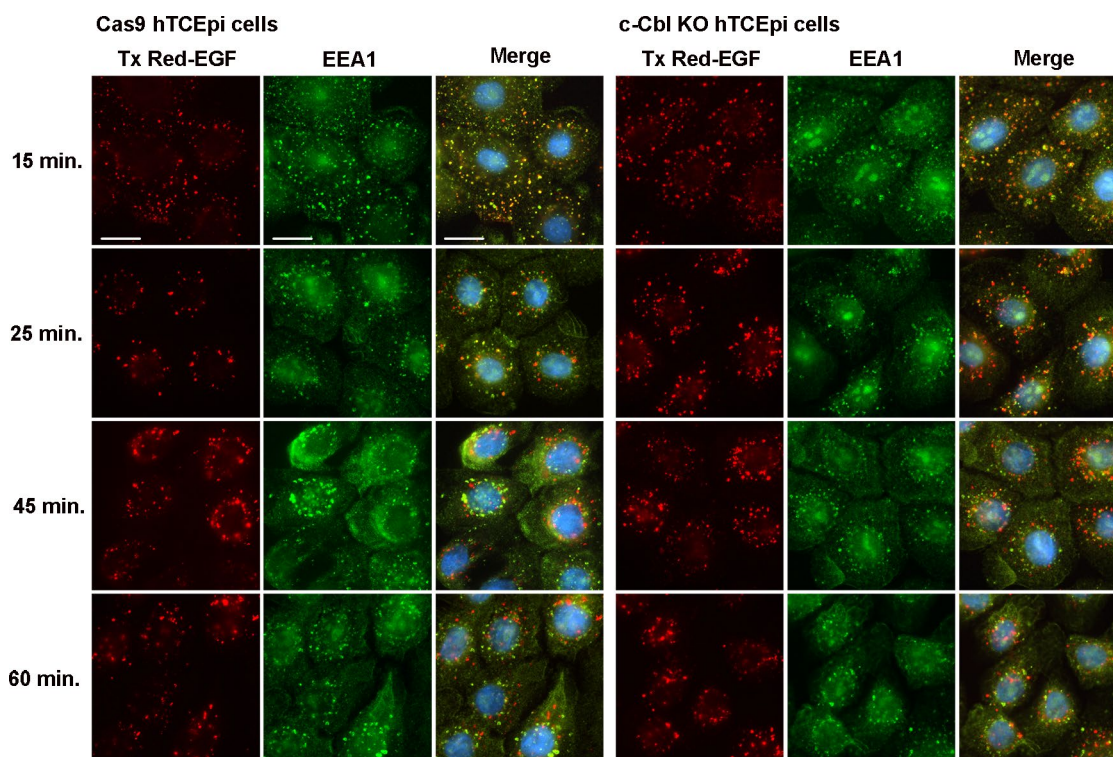

FIGURE 2 The EGF internalizes in the absence of c-Cbl. CAS9 expressing or c-Cbl knockout hTCEpi cells were plated on glass coverslips and grown to ~80% confluence. Cells were serum-starved and then incubated with 2 μg/ml of Texas Red-EGF (Invitrogen) for 10 min at 37°C to allow the internalization of ligand:receptor complexes. Extracellular fluorescently labeled ligand was removed by ice-cold citric acid washes. Cells were re-equilibrated in a neutral buffer and returned to 37°C growth media and 37°C. At the indicated time points, cells were fixed and processed for indirect immunofluorescence using a mouse monoclonal antibody against EEA1 (#C45B10, Cell Signaling) and an Alexa488 goat anti-mouse secondary antibody. Cells were mounted on microscope slides using Prolong with DAPI (Thermo Fisher Scientific). The micrographs indicate the localization of Texas Red-EGF (Red), EEA1 (green), and the nucleus (blue). Size bar = 10 μm. Shown is a representative experiment repeated at least three times
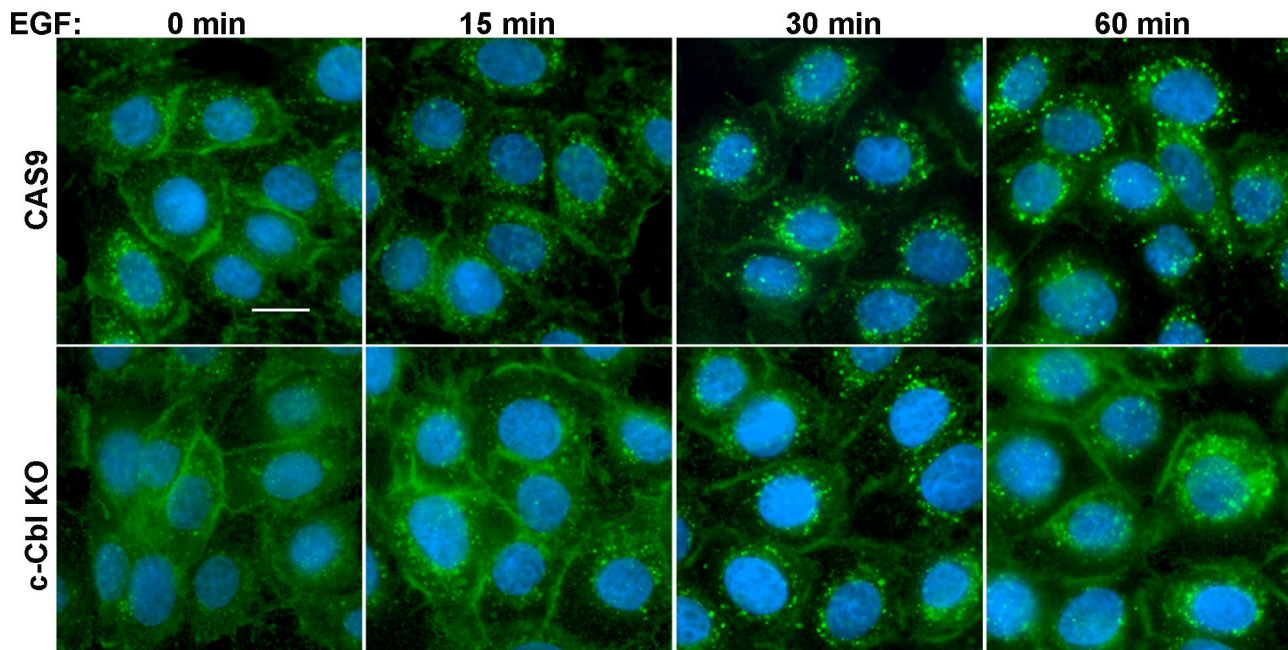
EGFR (green)/DAPI (blue)

FIGURE 3 EGFR distribution is altered by the knockout of c-Cbl. CAS9 expressing or c-Cbl knockout hTCEpi cells were plated on glass coverslips and grown to ~80% confluence. Cells were stimulated with 10 ng/ml of EGF for 0–60 min. At the indicated time point, cells were fixed and processed for indirect immunofluorescence using a mouse monoclonal antibody against the EGFR (Ab-1, EMD Millipore) and an Alexa488 goat anti-mouse secondary antibody. Coverslips were mounted on microscope slides using Prolong with DAPI (Thermo Fisher Scientific). Micrographs indicate the subcellular localization of the EGFR (green) and nucleus (blue). Size bar = 10 μm. Shown is a representative experiment repeated at least three times
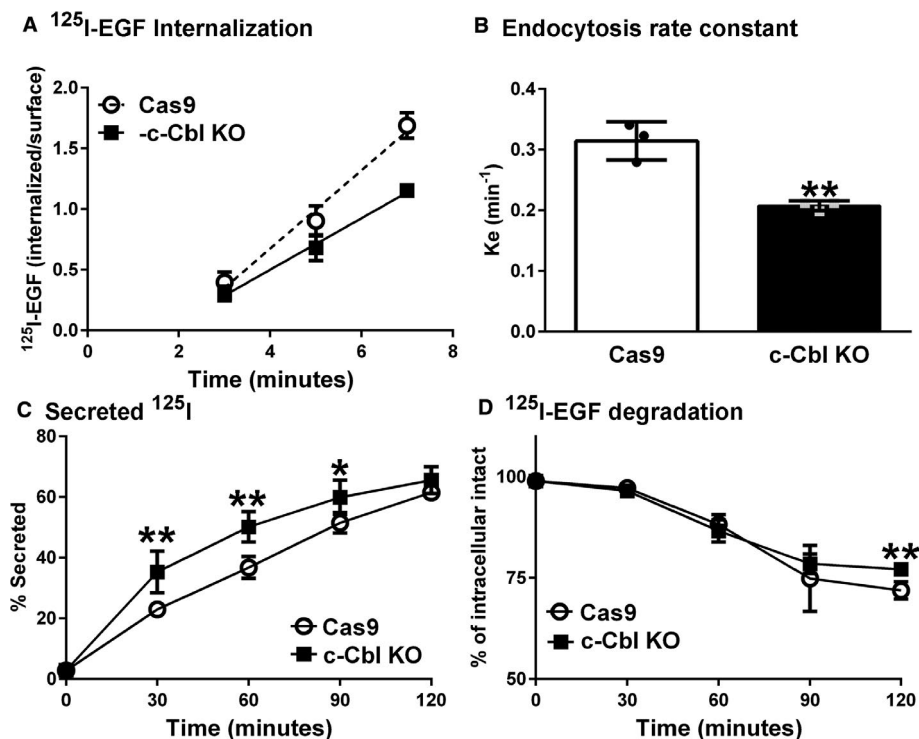

FIGURE 4 ^{125}I-EGF:EGFR endocytic trafficking is slowed in c-Cbl knockout cells. CAS9 expressing or c-Cbl knockout hTCEpi cells were plated in 35 mm dishes and grown to ~80% confluence. ^{125}I-EGF was used to monitor A–B) internalization, (C) recycling, and (D) degradation as described in Materials and Methods ($n = 3$). (A) The average (±SD) ratio of internalized ^{125}I-EGF/surface ^{125}I-EGF. (B) The average (±SD) calculated internalization constant (Ke) for each cell line. Data were analyzed using a paired t-test. (C) The average (±SD) percentage of total bound ^{125}I that was secreted into the media. Data were analyzed using an ANOVA with a Holm-Sidak post-analysis ($\alpha = 5.000\%$). (D) The average (±SD) percentage of intact ^{125}I-EGF. Data were analyzed using an ANOVA with a Holm-Sidak post-analysis ($\alpha = 5.000\%$). *$p < 0.10$; **$p < 0.05$
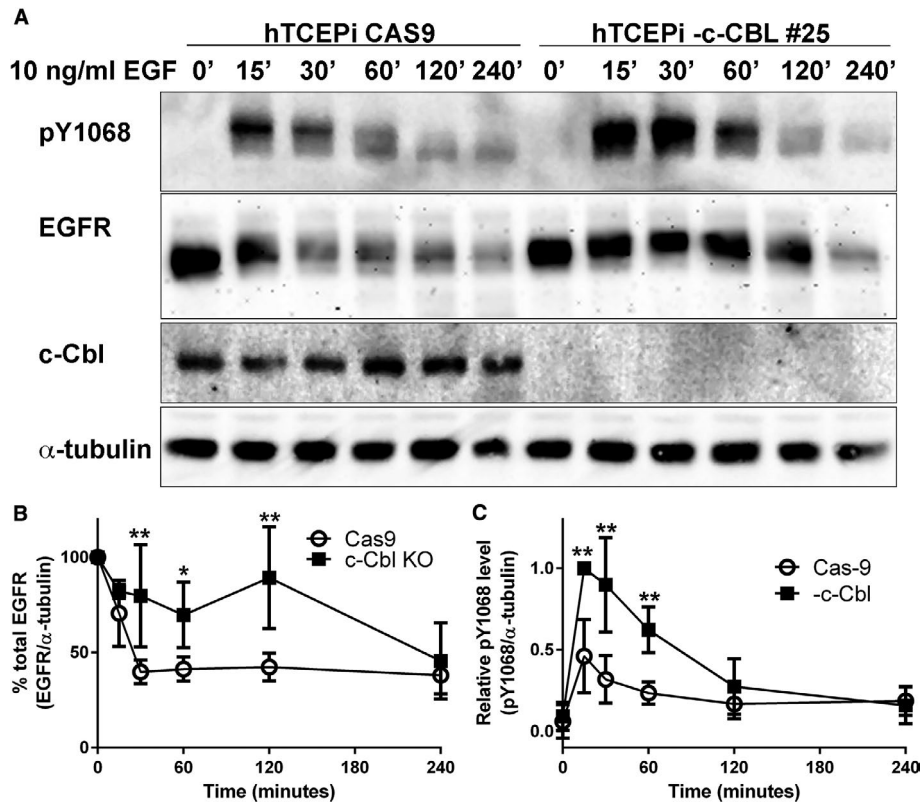
A

FIGURE 5 Knockdown of c-Cbl slows EGFR degradation and sustains ligand-mediated phosphorylation. Control (Cas-9) and c-Cbl knockout hTCEpi cells were serum-starved and treated with 10 ng/ml of EGF for the indicated amounts of time. (A) Cell lysates were prepared and equivalent amounts of protein (30 μg) were resolved by 7.5% SDS-PAGE. The resulting gel was transferred to nitrocellulose and immunoblotted for EGFR, phosphoEGFR (pY1068), c-Cbl and α-tubulin. Shown are representative immunoblots from an experiment that was performed three times using multiple c-Cbl knockout clones. (B and C) Immunoblots were quantified using NIH ImageJ. (B) The average (±SD) percentage of EGFR relative to α-tubulin and normalized to unstimulated levels ($t = 0$). (C) The average (±SD) percentage of phosphorylated EGFR (EGFR pY1068) relative to α-tubulin and normalized to the maximal phosphorylation ($t = 15$ min). Data were analyzed by an ANOVA with a Sidak-Bonferroni post-test analysis ($\alpha = 5.000\%$). *$p < 0.05$; **$p < 0.01$

dephosphorylation (Figure 5). The knockout of c-Cbl extended the half-life ($t_{1/2}$) nearly twofold as compared to control cells (CAS9 $t_{1/2} = 45$ min vs. c-Cbl KO $t_{1/2} = 87$, calculated from 0 to 60 min of EGF treatment) (Figure 5A and B). These changes in the rates of degradation were accompanied by a twofold increase in the magnitude of receptor phosphorylation, but, no significant change in the duration of receptor phosphorylation (Figure 5A and C).

Mitogen-activated protein kinase (MAPK) activity was used as a surrogate for intracellular signaling by the EGFR (Figure 6). A time course of EGF-stimulated MAPK phosphorylation revealed that the loss of c-Cbl has no effect on the magnitude of MAPK phosphorylation but extends the duration of signaling 2–3 h (Figure 6B).

4 | DISCUSSION

This study demonstrates that c-Cbl is not the only mediator of EGFR ubiquitylation in corneal epithelial cells. Even in the absence of detectable c-Cbl, ligand stimulation causes receptor ubiquitylation, albeit to a lesser extent than control cells (Figure 1B). These findings are consistent with reports from breast cancer-derived

BT20 cells,[27] as well as human duodenal carcinoma (HuTu-80) cells[37] and porcine arterial endothelial (PAE) cells[34] expressing exogenous EGFRs.

Since there are no detectable increases in expression of the other E3 ligases (Figure 1A), the residual ubiquitylation is not due to compensation by other endogenous E3 ligases. It is unclear if other E3 ligases provide functional redundancy, confer site-specific ubiquitylation, or regulate receptor ubiquitylation in a spatio-temporal specific manner. The latter two possibilities are consistent with each E3 ligase regulating specific components of receptor trafficking and signaling.

Our analysis of ligand and receptor trafficking combines single cell (fluorescence-based assays) and biochemical (radioligand and immunoblot analysis) approaches. Through these multi-faceted approaches, we observed that despite there being an incomplete loss of receptor ubiquitylation, those changes were sufficient to alter, but not completely block, endocytic trafficking of the ligand:receptor complex.

Using the Texas Red-EGF (Figure 2), we observe that the ligand:receptor complex can be internalized and its movement through the cell is indistinguishable from control cells. In this assay, the only ligand that was internalized is detected, as cell surface ligand is washed away using a citric acid wash. Once inside the cell,
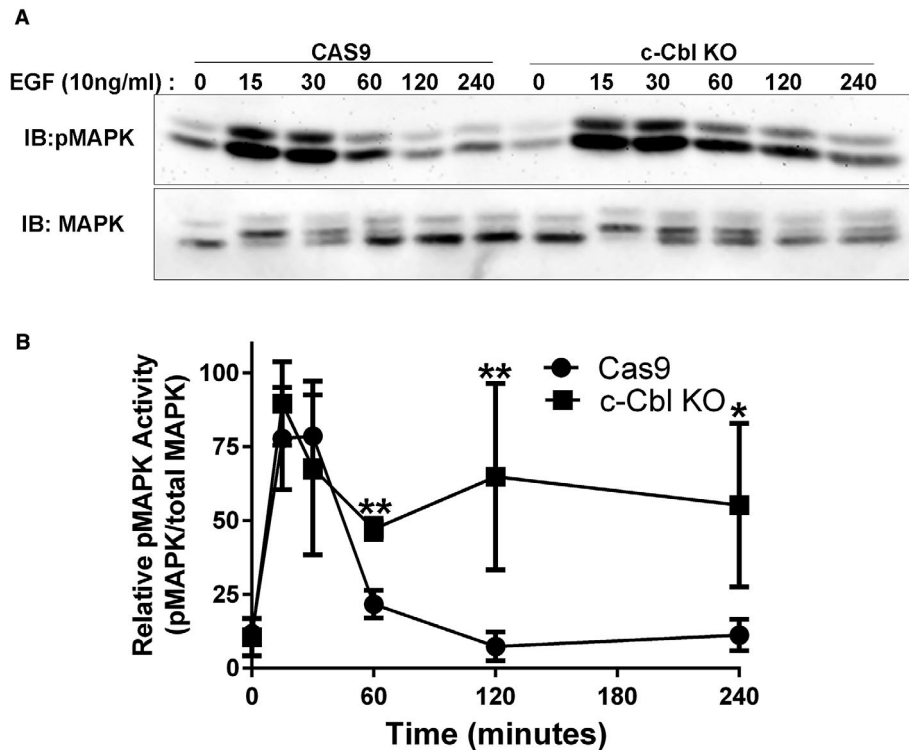
A

B

FIGURE 6 Knockdown of c-Cbl sustains EGFR-mediated MAPK phosphorylation. CAS9 expressing or c-Cbl knockout hTCEpi cells were serum-starved and treated with 10 ng/ml of EGF for the indicated amounts of time. (A) Cell lysates were prepared and equivalent amounts of protein (30 μg) were resolved by 10% SDS-PAGE. The resulting gel was transferred to nitrocellulose and immunoblotted for phosphoMAPK and total MAPK. Shown are representative immunoblots from an experiment that was performed three times using multiple c-Cbl knockout clones. (B) Immunoblots were quantified using NIH ImageJ. The average (±SD) relative phosphoMAPK activity (plotted as a ratio of phosphoMAPK/total MAPK). Levels are normalized to the maximal phosphorylation (t = 15 min). Data were analyzed with an ANOVA with a Sidak-Bonferroni post-test analysis (α = 5.000%, *$p < 0.05$; **$p < 0.01$)

the movement of the ligand is largely unchanged by the absence of c-Cbl. However, when the subcellular distribution of the receptor is examined (Figure 3), c-Cbl knockout cells clearly retain EGFRs on the cell surface 30–60 min after ligand stimulation.

These differences were quantified using radioligand assays. There was a 35% decrease in the rate of ligand:receptor endocytosis. Our data show the same trend as reported by Huang et al,[38] but we did not observe as robust of an effect from the c-Cbl knockout. This is likely due to the difference in cell line and level of receptor expression. In their studies, c-Cbl was transiently knocked down using siRNA in PAE cells expressing exogenous EGFRs.[38] The corneal epithelial cells used in these experiments express EGFRs at ~1.0 × 10^6 receptors/cell.[14]

The observation that c-Cbl deficient cells have increased ^{125}I secretion is consistent with the work of Eden et al.[24] They demonstrated that overexpressed ubiquitylation-deficient EGFRs internalized and then preferentially recycled from the early endosome. The increase in secreted [125]I (30–90 min after ligand treatment) (Figure 4C) is consistent with this finding. The rapid kinetics of ^{125}I secretion and the absence of any differences in degraded intracellular ^{125}I-EGF until 120 min post-endocytosis is consistent with the recycling of the ligand.

The slowed endocytosis and increased recycling are supported by a decreased rate of EGFR degradation (Figure 5B). Importantly, these changes in trafficking are sufficient to increase the magnitude of EGFR phosphorylation and sustain MAPK phosphorylation. Thus, the changes in trafficking have a biological consequence. Additional studies are needed to determine if these changes in signaling impact other downstream effectors (i.e., STAT3, PI3-K, Akt, PLCγ) or are unique to MAPK.

The different kinetic profiles in EGFR and MAPK activity reignite questions about the spatial regulation in receptor tyrosine kinase signaling. The loss of c-Cbl increases the magnitude of EGFR phosphorylation at relatively short time points (0–120 min), but MAPK signaling is sustained at longer time points (120–240 min). Although it is well established that there are differences in the kinetics of EGFR and MAPK activity, these data temporally separate the phosphorylation of each protein. The EGFR-mediated signaling to MAPK takes place well before the receptor becomes dephosphorylated or degraded.

Due to technical limitations, previous in vivo studies have only provided indirect evidence that antagonizing ubiquitylation will promote corneal epithelial wound healing and homeostasis.[39] However, this study provides direct evidence that the inhibition of c-Cbl can

prevent EGFR desensitization as indicated by the slowed rate of EGFR degradation and sustained receptor phosphorylation. Although inhibition of c-Cbl alone is sufficient to enhance EGFR signaling, it does not rule out the possibility that an even greater response could be achieved by antagonizing other E3 ligases, either alone or in tandem with c-Cbl antagonism.[27,40] However, in vivo studies are needed to determine the optimal level of EGFR ubiquitylation. A complete loss of EGFR ubiquitylation may be deleterious to the health of the corneal epithelium.

The role of c-Cbl as a negative regulator of EGFR signaling has been well established using cancer cells or cell lines engineered to express exogenous EGFRs. Its role in regulating endogenous EGFRs that contribute to tissue homeostasis has been less well studied. There has been a movement to pharmacologically target E3 ligases for the treatment of various pathological conditions,[41] including the restoration of damaged corneal epithelium. For these compounds to be successful therapeutic agents, it is important to understand the substrate specificity, what stages of membrane trafficking are impacted, and how cell-biology is affected. Although the inhibition of EGFR ubiquitylation in corneal epithelial cells has been shown to enhance tissue regeneration,[39] this study in corneal epithelial cells is the first to define the contribution of c-Cbl in controlling EGFR endocytic trafficking and signaling. We are now positioned to study the biological consequences using in vitro (cell proliferation, migration, viability) and in vivo (corneal epithelial development and wound healing).

Finally, c-Cbl is an E3 ligase for a number of other proteins. This initial study examines the EGFR because of its central role in corneal epithelial homeostasis. However, other c-Cbl substrates, including receptors (i.e., CSF1 receptor, EphA2, FGF receptor 2, VEGF receptor 2) and effectors (i.e., PLCγ1, Abl),[42–47] are expressed in the corneal epithelium[48–50] and contribute to tissue homeostasis. In the future, this cell line can be used to assess the role of the ubiquitylation of other substrates in corneal epithelial homeostasis.

5 | CONCLUSION

We report that knockout of c-Cbl was sufficient to disrupt EGFR endocytic trafficking in corneal epithelial cells. Our data demonstrate that the knockout of c-Cbl is not sufficient to completely block ligand-mediated EGFR ubiquitylation. This reduction in ubiquitylation is sufficient to delay receptor endocytosis and degradation, as well as, enhance EGFR phosphorylation and signaling. Thus, antagonizing c-Cbl is a viable strategy for enhancing corneal epithelial homeostasis and wound healing.

DISCLOSURE
The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS
Participated in research design: Crotchett, B.L.M., Ceresa, B.P. Conducted experiments: Crotchett, B.L.M., Ceresa, B.P. Performed data analysis: Crotchett, B.L.M., Ceresa, B.P. Wrote or contributed to the writing of the manuscript: Crotchett, B.L.M., Ceresa, B.P.

ETHICAL APPROVAL
There were no ethical considerations or approvals required for this manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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