

# Ubiquitylation of the chemokine receptor CCR7 enables efficient receptor recycling and cell migration

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## Summary

The chemokine receptor CCR7 is essential for lymphocyte and dendritic cell homing to secondary lymphoid organs. Owing to the ability to induce directional migration, CCR7 and its ligands CCL19 and CCL21 are pivotal for the regulation of the immune system. Here, we identify a novel function for receptor ubiquitylation in the regulation of the trafficking process of this G-protein-coupled seven transmembrane receptor. We discovered that CCR7 is ubiquitylated in a constitutive, ligand-independent manner and that receptor ubiquitylation regulates the basal trafficking of CCR7 in the absence of chemokine. Upon CCL19 binding, we show that internalized CCR7 recycles back to the plasma membrane via the trans-Golgi network. An ubiquitylation-deficient CCR7 mutant internalized normally after ligand binding, but inefficiently recycled in immune cells and was transiently retarded in the trans-Golgi network compartment of HEK293 transfectants. Finally, we demonstrate that the lack of CCR7 ubiquitylation profoundly impairs immune cell migration. Our results provide evidence for a novel function of receptor ubiquitylation in the regulation of CCR7 recycling and immune cell migration.

**Key words:** Ubiquitylation, Receptor trafficking, Recycling, Cell migration, Chemokine receptor

## Introduction

Chemokine receptors represent a family of seven-transmembrane spanning, G-protein-coupled receptors which interact with chemotactic cytokines termed chemokines. Chemokine receptor triggering results in the onset of complex intracellular signaling cascades leading to cell polarization and migration towards the chemokine source (Thelen and Stein, 2008). Cell migration is pivotal for the induction of immune responses and hence is tightly regulated (Bromley et al., 2008). The chemokine receptor CCR7 is mainly expressed on naïve lymphocytes and mature dendritic cells and responsible for the homing of these cells to secondary lymphoid organs where dendritic cells present the acquired antigens to T cells (Förster et al., 2008). CCR7 has two known ligands, CCL19 and CCL21, which are both constitutively expressed by stroma cells in lymphoid tissues (Luther et al., 2000). Stimulation of CCR7 with either ligand was shown to similarly induce cell migration of primary T cells (Bardi et al., 2001; Schaeuble et al., 2011) and 300-19 transfectants (Otero et al., 2006; Willimann et al., 1998), G protein activation in H9 T cells (Kohout et al., 2004), ERK-1/2 phosphorylation in HEK293 transfectants (Kohout et al., 2004), and calcium mobilisation in primary T cells as well as various T cell lines (CEM, HuT78, H9) and HEK293 or 300-19 transfectants (Bardi et al., 2001; Kohout et al., 2004; Otero et al., 2008; Otero et al., 2006). In contrast, only CCL19 triggering resulted in CCR7 phosphorylation on serine/threonine residues in HEK293 transfectants (Kohout et al., 2004) and  $\beta$ -arrestin2 binding (Byers et al., 2008; Comerford et al., 2006;

Kohout et al., 2004), which subsequently leads to profound receptor internalization in primary T cells and monocyte-derived dendritic cells (Bardi et al., 2001; Otero et al., 2006), as well as various cell lines and transfectants (Bardi et al., 2001; Comerford et al., 2006; Otero et al., 2006). Interestingly, CCR7 internalization was also observed by CCL21, but only at higher chemokine concentration and receptor endocytosis was about five times less efficient compared to CCL19 (Byers et al., 2008; Otero et al., 2006). Recently, a biased ligand model for CCL19 and CCL21 was proposed where both chemokines are able to recruit  $\beta$ -arrestin2 to CCR7 in HEK293 transfectants by activating G-protein-coupled receptor kinase 6 (GRK6), resulting in ERK-1/2 phosphorylation, whereas only CCL19 was able to additionally activate GRK3 leading to  $\beta$ -arrestin2-dependent CCR7 internalization (Zidar et al., 2009). Using primary T cells and HEK293 transfectants, we recently demonstrated that CCR7 together with CCL19 are jointly internalized through clathrin-coated pits and subsequently transported to early endosomes (Otero et al., 2006). Internalized CCR7 recycled back to the plasma membrane of primary T cells as well as (transfected) cell lines (Bardi et al., 2001; Otero et al., 2006) to re-participate in cell migration, whereas CCL19 was sorted to lysosomes for degradation (Otero et al., 2006).

Ubiquitin is a small protein modifier with a variety of cellular functions that conjugates to different target proteins through a covalent bond between the C-terminal glycine of ubiquitin and the side chains of lysine on target proteins. Target proteins can either be monoubiquitylated at a single lysine residue, multiubiquitylated

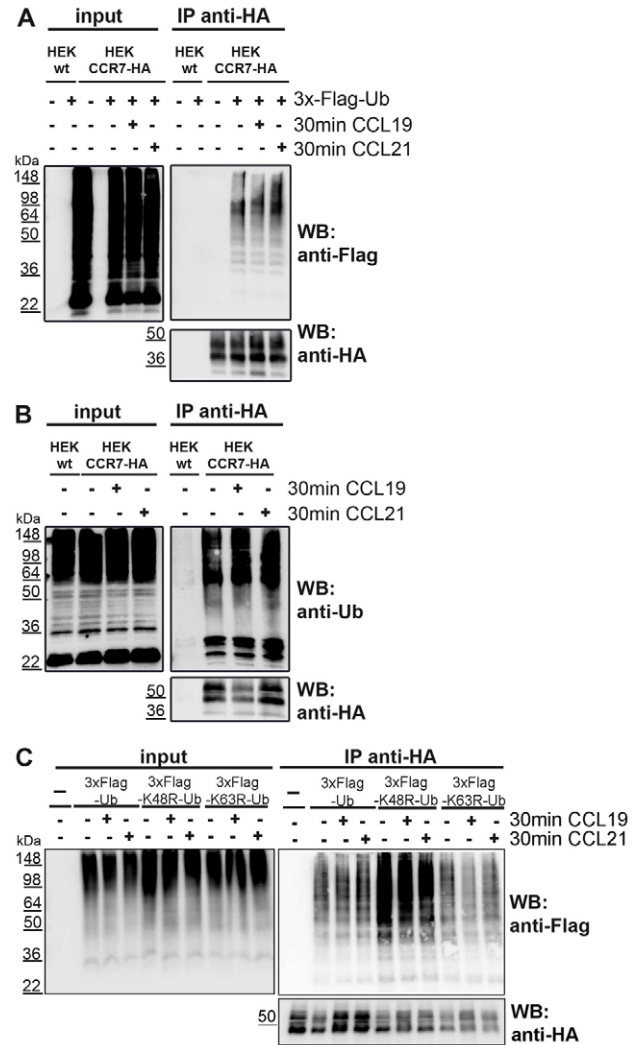
where single ubiquitin molecules are attached to multiple lysines, or polyubiquitylated where additional ubiquitin molecules are conjugated to different lysine residues of protein-attached ubiquitins (Ikeda et al., 2010; Mukhopadhyay and Riezman, 2007). The different kinds of ubiquitin modifications already indicate the distinct physiological function of each ubiquitylation. K48-polyubiquitylation, for instance, is the most common modification leading predominantly to proteasomal degradation of the target protein (Hershko and Ciechanover, 1998). Mono- and multiubiquitylation are mainly involved in DNA repair, cell-cycle progression, apoptosis, autophagy, virus budding or receptor endocytosis (Ikeda et al., 2010; Ikeda and Dikic, 2008; Mukhopadhyay and Riezman, 2007). That ubiquitylation can regulate transmembrane receptor trafficking was first described in yeast (Hicke and Riezman, 1996; Kölling and Hollenberg, 1994). More recent studies in mammalian cells demonstrated that ubiquitylation of transmembrane receptors, including GPCRs, does not primarily serve as an endocytosis signal, but rather targets internalized receptors to degradative cell compartments (Huang et al., 2007; Mukhopadhyay and Riezman, 2007; Shenoy et al., 2007).

So far, ubiquitylation of chemokine receptors was demonstrated for CXCR4 (Borroni et al., 2010; Marchese and Benovic, 2001; Marchese et al., 2003). Upon CXCL12 binding, CXCR4 was ubiquitylated by the E3 ubiquitin ligase AIP4, resulting in lysosomal sorting and subsequent degradation of CXCR4 (Marchese and Benovic, 2001; Marchese et al., 2003). Interestingly, CXCL12 was shown to recruit the de-ubiquitylation enzyme USP14 to CXCR4, thereby regulating CXCR4 degradation and cell migration (Mines et al., 2009). In addition, depletion of the de-ubiquitylation enzyme USP8 was found to stabilize CXCR4 surface expression without affecting receptor ubiquitylation, indicating a role of USP8 in CXCR4 trafficking and degradation (Berlin et al., 2010). In contrast, lysosomal degradation of the chemokine receptors CXCR2 and CXCR3 was independent of receptor ubiquitylation (Baugher and Richmond, 2008; Meiser et al., 2008). Whereas ligand-induced sorting of CXCR2 to lysosomes was mediated by Rab7 (Fan et al., 2003), CXCR3 underwent constitutive lysosomal sorting and degradation in the absence of ligands (Meiser et al., 2008). The role of ubiquitylation in function and trafficking of the homing chemokine receptor CCR7 has not been addressed until the present study.

## Results

### The chemokine receptor CCR7 is constitutively ubiquitylated

Ubiquitylation of transmembrane receptors can act as endocytosis or sorting signal (Mukhopadhyay and Riezman, 2007). The chemokine receptor CCR7 is rapidly internalized upon CCL19 binding, whereas about 80% of the receptor remains at the plasma membrane upon CCL21 activation (Byers et al., 2008; Otero et al., 2006). In order to investigate whether ubiquitylation regulates CCR7 trafficking, we stably expressed human CCR7 with a C-terminal HA tag in HEK293 cells. These cells were transiently transfected with a 3xFlag-tagged ubiquitin construct and incubated in the absence or presence of either CCR7 ligand for 30 min at 37°C. CCR7 was immunoprecipitated from cell lysates and ubiquitylated CCR7 detected by western blot analysis using an anti-Flag antibody. We found that CCR7 was constitutively ubiquitylated (Fig. 1A). Ubiquitylated CCR7 appeared as a smear at higher molecular masses, indicating



**Fig. 1. CCR7 is constitutively polyubiquitylated.** (A) Non-transfected, wild-type (wt) HEK293 or HEK293 cells stably expressing CCR7-HA were transiently transfected with 3xFlag-ubiquitin. 48 h after transfection, cells were either stimulated for 30 min with 1  $\mu$ g/ml of the indicated chemokine or left untreated. Subsequently, cells were lysed and HA-tagged CCR7 was immunoprecipitated (IP) using anti-HA-agarose. CCR7 and receptor-associated ubiquitin were detected with HRP-coupled anti-HA and anti-Flag antibodies. Cell lysates were immunoblotted (WB) for ubiquitin to control for differences in transfection efficiency (input). A representative blot out of four independent experiments is shown. (B) HA-tagged CCR7 was immunoprecipitated from chemokine-stimulated or non-stimulated HEK293 cells transiently transfected with 3xFlag-ubiquitin, and endogenous ubiquitin attached to CCR7 was visualized using a monoclonal anti-ubiquitin antibody. (C) HEK293-CCR7-HA cells were transiently transfected with either 3xFlag-ubiquitin, 3xFlag-K48R-ubiquitin or 3xFlag-K63R-ubiquitin, stimulated with the indicated ligands for 30 minutes, lysed and CCR7 was immunoprecipitated using anti-HA-agarose. Ubiquitylated CCR7 was detected by immunoblotting using the anti-Flag antibody M2 conjugated to HRP. Similar results were obtained in four independent experiments.

receptor polyubiquitylation. Surprisingly, CCR7 ubiquitylation only slightly increased after stimulation with either ligand, CCL19 or CCL21. The signal was specific as no ubiquitin smear was detected in immunoprecipitates derived from cells lacking CCR7. We confirmed the ubiquitylation of CCR7 with

endogenous ubiquitin (Fig. 1B). Conjugation of endogenous ubiquitin to CCR7 was again observed in non-stimulated, as well as chemokine stimulated cells. Due to the lack of appropriate antibodies, we were unable to assess ubiquitylation of endogenous CCR7.

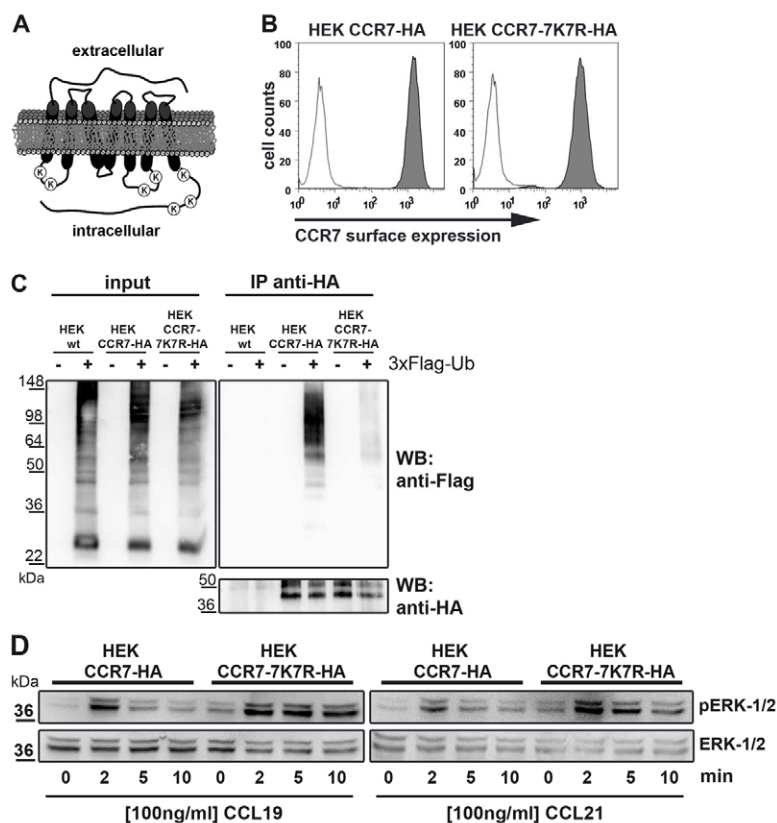
Conjugation of ubiquitin to a target protein requires three different enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase, of which the latest provides the specificity as it recognizes the substrate (Nagy and Dikic, 2010). Only a few E3 ligases are described for integral membrane proteins, including AIP4, which ubiquitylates the chemokine receptor CXCR4 (Marchese et al., 2003). In an attempt to identify the E3 ubiquitin ligase for CCR7, we knocked down the expression of the most promising candidates by siRNA, including AIP4, NEDD4-L, NEDD4 and Cbl-b (Georgieva et al., 2011; Huang et al., 2010; Marchese et al., 2003; Pennock and Wang, 2008). However, neither of these turned out to be the major E3 ligase for CCR7 ubiquitylation (supplementary material Fig. S1).

We next investigated on the ubiquitin linkage to gain insights into the function of this receptor modification. K48-linked polyubiquitin chains mainly target proteins to proteasomal degradation, whereas K63-linked ubiquitylation often serves as signal for receptor endocytosis (Mukhopadhyay and Riezman, 2007). Thus, we generated two ubiquitin mutants that prevent K48-mediated (K48R-Ub) or K63-mediated (K63R-Ub) ubiquitylation. As shown in Fig. 1C, K48R-Ub preferentially attached to CCR7 compared to K63R-Ub and wild-type ubiquitin, indicating that ubiquitin linkage via K48 is not the preferred one for CCR7 ubiquitylation.

### An ubiquitylation-deficient CCR7 mutant is expressed at the plasma membrane and elicits chemokine-mediated ERK activation

So far we cannot fully rule out that an ubiquitylated protein associated with CCR7 might account for the smear observed in Fig. 1. To address this possibility and to identify the function of CCR7 ubiquitylation, we created a lysineless receptor mutant. Pursuant to the topology prediction by Swiss-Prot ([www.expasy.org/sprot/](http://www.expasy.org/sprot/)), CCR7 harbours seven cytoplasmic lysine residues that could potentially be ubiquitylated (Fig. 2A). We cloned HA-tagged CCR7-7K7R in which all seven lysine residues were replaced by arginines and stably expressed it in HEK293 cells. CCR7-7K7R-HA was properly inserted into the plasma membrane and surface expression levels were comparable to wild-type CCR7 as determined by flow cytometry (Fig. 2B). Immunoprecipitation studies in cells expressing either HA-tagged CCR7 or CCR7-7K7R together with 3xFlag-ubiquitin revealed a virtually complete loss of ubiquitylation in the lysineless CCR7 mutant (Fig. 2C). To gain further evidence that CCR7 is directly ubiquitylated, we subjected affinity purified, trypsin-digested CCR7 to mass spectrometry analysis. Analysis of the multiple fragmentation spectra clearly identified a direct ubiquitylation site as manifested by a di-glycine modification (derived from ubiquitin) on lysine 342 of CCR7 (supplementary material Fig. S2). We cannot exclude that additional lysine residues might be ubiquitylated as the sequences identified by mass spectrometry covered about 15–26% of the total CCR7 sequence. However, our data prove that CCR7 is directly ubiquitylated.

As chemokine-mediated activation of the MAP-kinases ERK-1/2 is one of the most established, early signaling events, we next



**Fig. 2. The lysineless ubiquitylation-deficient CCR7-7K7R mutant elicits ERK phosphorylation.** (A) Schematic representation of CCR7. Predicted intracellular lysine residues, representing putative ubiquitin conjugation sites, are highlighted and were mutated to arginine to generate the lysineless CCR7-7K7R mutant. (B) HEK293 cells stably expressing either CCR7-HA or CCR7-7K7R-HA were stained with a biotin-conjugated CCR7-specific antibody followed by PE-labeled streptavidin. Cell surface expression was monitored by flow cytometry. Solid lines depict control staining with PE-streptavidin in the absence of primary antibody. Experiments were reproduced at least four times revealing similar results. (C) HEK293 cells, either non-transfected (wild type, wt) or expressing CCR7-HA or 7K7R-CCR7-HA, respectively, were transiently transfected with 3xFlag-ubiquitin. 48 h later, cells were lysed and HA-tagged CCR7 was immunoprecipitated using anti-HA-agarose. Receptor ubiquitylation was detected with the anti-Flag antibody M2. (D) HEK293 cells expressing CCR7-HA or CCR7-7K7R-HA were stimulated for indicated times with 100 ng/ml chemokine at 37°C and subsequently lysed. Total cell lysates were immunoblotted for phospho-ERK-1/2 (pERK-1/2). Reprobing of the stripped blot with a total ERK-1/2 antibody served as loading control.

investigated whether CCR7 ubiquitylation influences ERK-1/2 activation. Therefore, we stimulated HEK293 transfectants with either CCL19 or CCL21 for different time points and determined ERK-1/2 phosphorylation by western blotting. As depicted in Fig. 2D, CCR7-7K7R expressing cells elicited a profound chemokine-mediated ERK-1/2 phosphorylation which was more sustained compared to cells expressing wild-type CCR7. No chemokine-mediated ERK-1/2 activation, however, was detectable anymore after 20 min of stimulation, neither in CCR7 nor in CCR7-7K7R expressing cells (data not shown). As sustained ERK-1/2 activation in CCR7-7K7R could result from an enhanced affinity of the chemokine, we incubated cell transfectants with increasing concentrations of CCL19-Fc (Otero et al., 2006) and determined binding by flow cytometry. However, CCL19-Fc bound with similar affinities to CCR7 ( $K_D$ :  $21.1 \pm 5.0$  nM) and CCR7-7K7R ( $K_D$ :  $40.9 \pm 6.7$  nM).

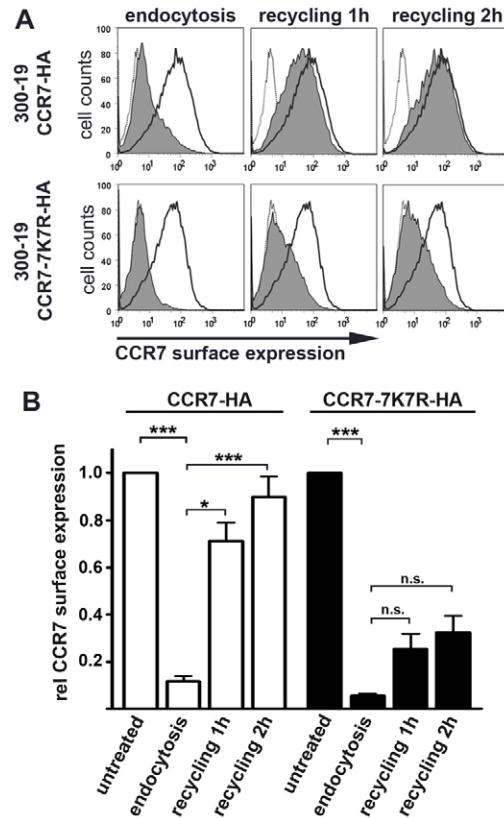
### Ubiquitylation of CCR7 is critical for receptor recycling in 300-19 B cells

Next, we investigated the role of ubiquitylation in CCR7 trafficking. To this end, we generated immune cell lines expressing the two CCR7 forms. We used the commonly used pre-B cell line 300-19 that does not endogenously express CCR7 (Otero et al., 2008; Otero et al., 2006; Willimann et al., 1998). Stimulation of CCR7 expressing 300-19 cells with CCL19 provoked rapid and profound receptor endocytosis (Fig. 3), confirming previous studies using cell lines and primary T cells (Bardi et al., 2001; Comerford et al., 2006; Otero et al., 2008; Otero et al., 2006). Interestingly, CCL19-mediated CCR7-7K7R endocytosis was comparable to that of CCR7 (Fig. 3). CCR7 internalization by CCL21 occurs only at elevated chemokine concentrations and is about five times less efficient compared to CCL19 leaving about 80% of the receptor at the cell surface upon CCL21 stimulation (Byers et al., 2008; Otero et al., 2006). Similarly, CCL21-mediated endocytosis of CCR7-7K7R was about 4.5 times less efficient than CCL19-mediated internalization (data not shown). These results provide evidence that ubiquitylation is not a signal to induce CCR7 internalization.

To determine receptor recycling, chemokine-triggered cells were washed extensively and incubated for 1 or 2 h in the absence of ligands to permit receptor re-expression at the cell surface. As expected, CCR7 recycled back to the plasma membrane reaching again 70% cell surface expression after 1 h (Fig. 3; Otero et al., 2008; Otero et al., 2006). In contrast, CCR7-7K7R failed to efficiently recycle back to the plasma membrane after removing of the ligand (Fig. 3). Also after 2 h, only a minor fraction of lysineless CCR7 re-appeared at the cell surface (Fig. 3), indicating that impaired ubiquitylation strongly impaired and decelerated the recycling process in immune cells.

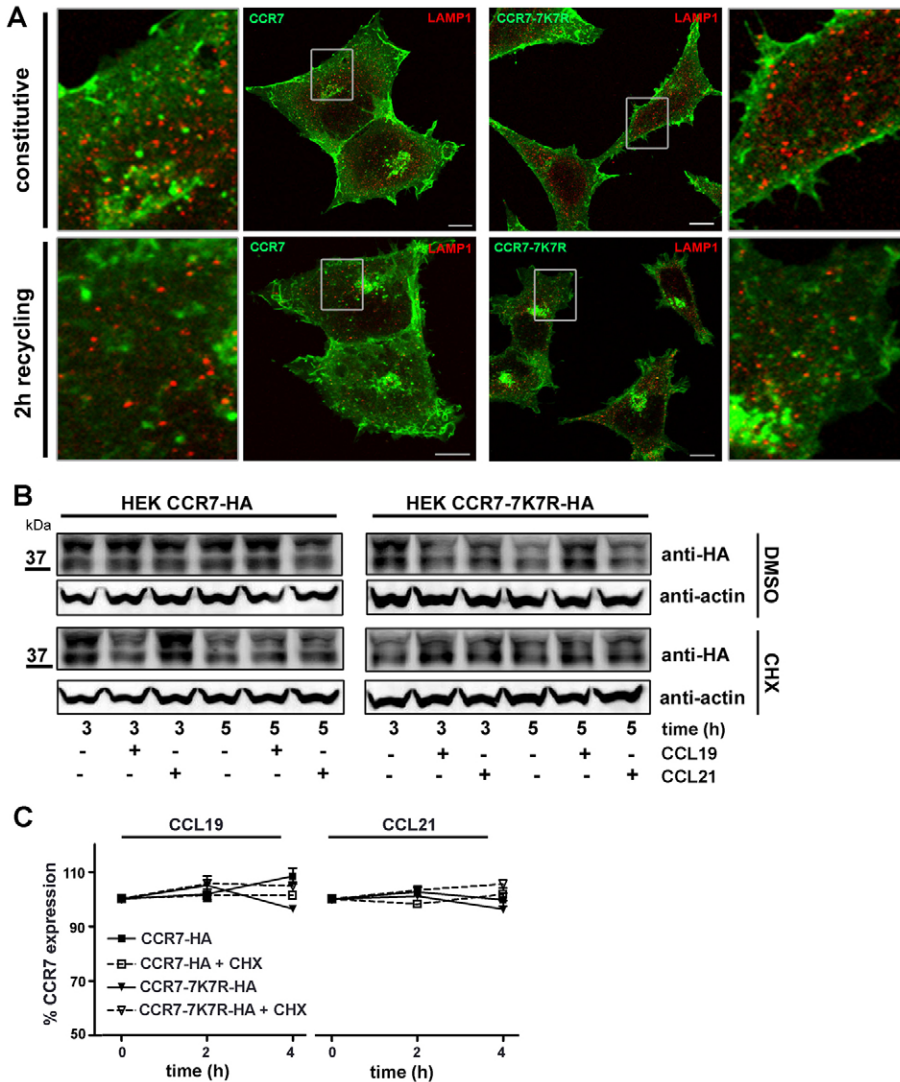
### Efficient CCR7 ubiquitylation is important for constitutive receptor trafficking

As CCR7-7K7R showed abnormal trafficking, we generated GFP-tagged CCR7 and its lysineless mutant to visualize their intracellular localization in HEK293 cells, a cell line routinely used to study chemokine receptor trafficking. We have shown previously that C-terminal fusion of GFP to CCR7 has no adverse effects on receptor signaling and trafficking (Otero et al., 2006). As expected, ligand-induced receptor endocytosis was comparable in HEK293 cells expressing CCR7-GFP and CCR7-7K7R-GFP (supplementary material Fig. S3A,B). About 50% of internalized



**Fig. 3. Ubiquitylation-deficient CCR7 displays strongly impaired receptor recycling in 300-19 B cells.** 300-19 B cells stably expressing similar amounts of HA-tagged CCR7 or CCR7-7K7R were incubated with 2  $\mu$ g/ml CCL19 for 30 min to induce receptor endocytosis. For receptor recycling, CCL19-stimulated cells were washed extensively to remove unbound ligand and further incubated for 1 or 2 h in the absence of chemokine, facilitating the recycling of the receptor back to the plasma membrane. Cells were placed in cold PBS and surface expression of CCR7 was assessed by staining with a specific antibody at 4°C and analyzed by flow cytometry. (A) Histograms of a representative experiment showing basal receptor expression before chemokine stimulation (black bold line) together with either endocytosed or recycled receptor levels (gray). Thin gray lines represent isotype control staining. (B) Relative CCR7 surface expression after endocytosis and recycling as percentage of untreated cells. Mean values  $\pm$  s.e.m. of at least nine independent experiments are depicted as the relative expression of untreated cells. Statistical significances were analyzed performing a Kruskal–Wallis test (nonparametric ANOVA) with Dunn post test. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; n.s. not significant.

CCR7-GFP recycled back to the plasma membrane after 1 h of ligand removal (supplementary material Fig. S3B). CCR7 trafficking is less efficient in HEK293 cells compared to 300-19 cells and primary lymphocytes, as described previously (Bardi et al., 2001; Otero et al., 2006). CCR7-7K7R-GFP inefficiently recycled after 1 h but steadily re-appeared at the plasma membrane after 2 and 4 h (supplementary material Fig. S3B). These data indicate that trafficking efficiencies vary for an unknown reason among different cell types. Next, we investigated lysosomal localization of CCR7 and its lysineless mutant by confocal microscopy, as ubiquitylation is a known lysosomal targeting signal. Under basal, chemokine-free conditions, neither CCR7-GFP nor CCR7-7K7R-GFP colocalized with the lysosomal marker LAMP1 (Fig. 4A). CCR7-GFP was also not detected in the

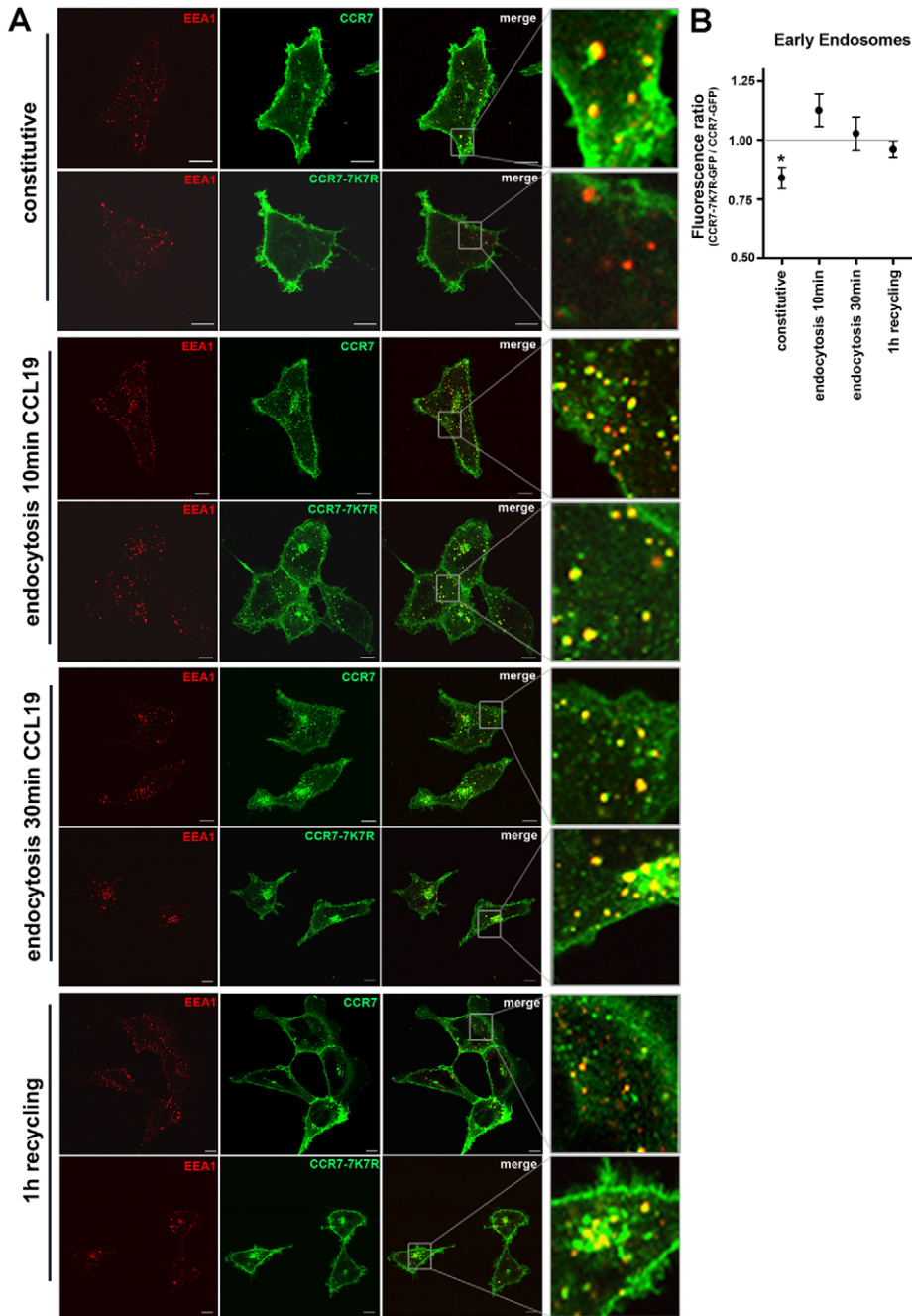


**Fig. 4. Ubiquitylation of CCR7 is not a sorting signal to lysosomes for degradation.** (A) CCR7-GFP or CCR7-7K7R-GFP (shown in green) in a non-stimulated, constitutive state and after recycling, was visualized in HEK293 cells by confocal microscopy. Lysosomal compartments were stained using an anti-LAMP1 antibody (in red). Images are representative of many cells examined in three separate experiments. Scale bars: 10  $\mu$ m. (B) HEK293 cells stably expressing HA-tagged CCR7 or CCR7-7K7R were treated either with 50  $\mu$ g/ml cycloheximide (CHX) or its solvent control (DMSO) and stimulated with 2  $\mu$ g/ml CCL19 or CCL21, respectively. Cells were lysed after the indicated time periods and CCR7 expression was determined by western blotting using an anti-HA antibody. Blots were stripped and reprobbed with an anti-actin antibody to ensure equal protein loading. A representative result out of three independent experiments is depicted. (C) HEK293 cells stably transfected with CCR7-GFP or CCR7-7K7R-GFP were treated with 2  $\mu$ g/ml CCL19 or CCL21 for the indicated time periods in the absence or presence of CHX. Fluorescence of GFP was assessed by flow cytometry. Mean values  $\pm$  s.e.m. of at least three independent experiments are shown.

lysosomal compartment after CCL19-mediated receptor internalization and subsequent incubation in the absence of ligands (Fig. 4A), confirming previous observations (Otero et al., 2006). Similarly, no colocalization of CCR7-7K7R-GFP with LAMP1 was detectable after CCL19 stimulation (Fig. 4A), indicating that the lysineless receptor variant is also not degraded in lysosomes and that ubiquitylation is not a lysosomal targeting signal for CCR7. To further support this finding, we investigated receptor degradation by western blot analysis in cells additionally treated with cycloheximide to prevent receptor neosynthesis. As depicted in Fig. 4B, neither HA-tagged CCR7 nor CCR7-7K7R was degraded upon chemokine stimulation for different time points. In contrast, transiently transfected HA-tagged FAT10, a short-lived ubiquitin-like modifier, was no longer detectable after 5 h of cycloheximide treatment thus validating the conditions of monitoring protein degradation by cycloheximide chase (data not shown). Furthermore, we also found no evidence for ligand-induced receptor degradation by measuring the fluorescence of GFP-tagged receptors by flow cytometry (Fig. 4C).

Thus, we further investigated the fate of CCR7 and its lysineless mutant after initial internalization steps. We described

previously that CCR7 rapidly internalized after CCL19 triggering via clathrin-coated pits and subsequently localized in transferrin-positive endosomes (Otero et al., 2006). Here, we demonstrate that a small but significant fraction of CCR7-GFP colocalized with the early endosomal marker EEA1 already in the absence of ligand (Fig. 5A). The amount of CCR7-GFP containing structures was very low but consistent in all cells expressing CCR7-GFP analyzed. Strikingly, in the absence of chemokine, the lysineless CCR7 was hardly detectable in early endosomal vesicles. Under constitutive conditions, the amount of EEA1 positive structures, containing the lysineless receptor mutant was reduced in comparison to wild-type receptor expressing cells (Fig. 5B). These observations suggest that one function of ubiquitylation is to control CCR7 steady-state surface expression. Next, we determined early endosome localization of the receptor after 10 and 30 min of CCL19 treatment. Substantial receptor internalization and localization in early endosomal vesicles was detected for both, CCR7 and its lysineless mutant (Fig. 5A). To investigate the storage period of the chemokine receptors in the early endosomal compartment, we treated the cells for 30 min with CCL19, removed the ligand and incubated the cells in the absence of chemokines. Some



**Fig. 5. Constitutive receptor trafficking, but not chemokine-mediated initial internalization steps, is impaired in ubiquitylation-deficient CCR7.** (A) HEK293 cells stably expressing either CCR7-GFP or CCR7-7K7R-GFP (shown in green) were either left untreated (constitutive) or incubated for 10 or 30 min with CCL19. For receptor recycling, cells were incubated for 30 min with CCL19, extensively washed to remove unbound ligand and incubated in chemokine-free medium for an additional hour to permit receptor recycling back to the plasma membrane. Subsequently cells were fixed, permeabilized, immunostained for EEA1 (in red) and viewed using confocal microscopy. Images are representative of many cells examined in three separate experiments. Scale bars: 10  $\mu$ m. (B) For quantification, mean GFP fluorescence in EEA1-positive compartments was determined and normalized to the overall mean GFP fluorescence of the cell as described in the Materials and Methods section. The ratio of these values derived from CCR7-7K7R-GFP- and CCR7-GFP-expressing cells is shown as the fluorescence ratio. Data were statistically evaluated using the two-tailed Mann-Whitney test. \* $P$ <0.05.

CCR7-GFP still colocalized with EEA1 after 1 h (Fig. 5A) and 4 h (supplementary material Fig. S3C), but the proportion was reduced compared to the initial 30 min after receptor triggering. Furthermore, CCR7-GFP was additionally found in globular structures that did not stain for EEA1 any more, which presumably represent recycling compartments. The amount of EEA1 positive, CCR7-7K7R-GFP-containing structures left after 1 h (Fig. 5A) and 4 h (supplementary material Fig. S3C) of ligand deprivation was also reduced; but a profound accumulation of CCR7-7K7R-GFP was observed in the perinuclear region of the cell. To test whether binding of CCL19 to CCR7 and its mutant is differentially sensitive to the reduced pH of endosomes, we performed CCL19-Fc binding

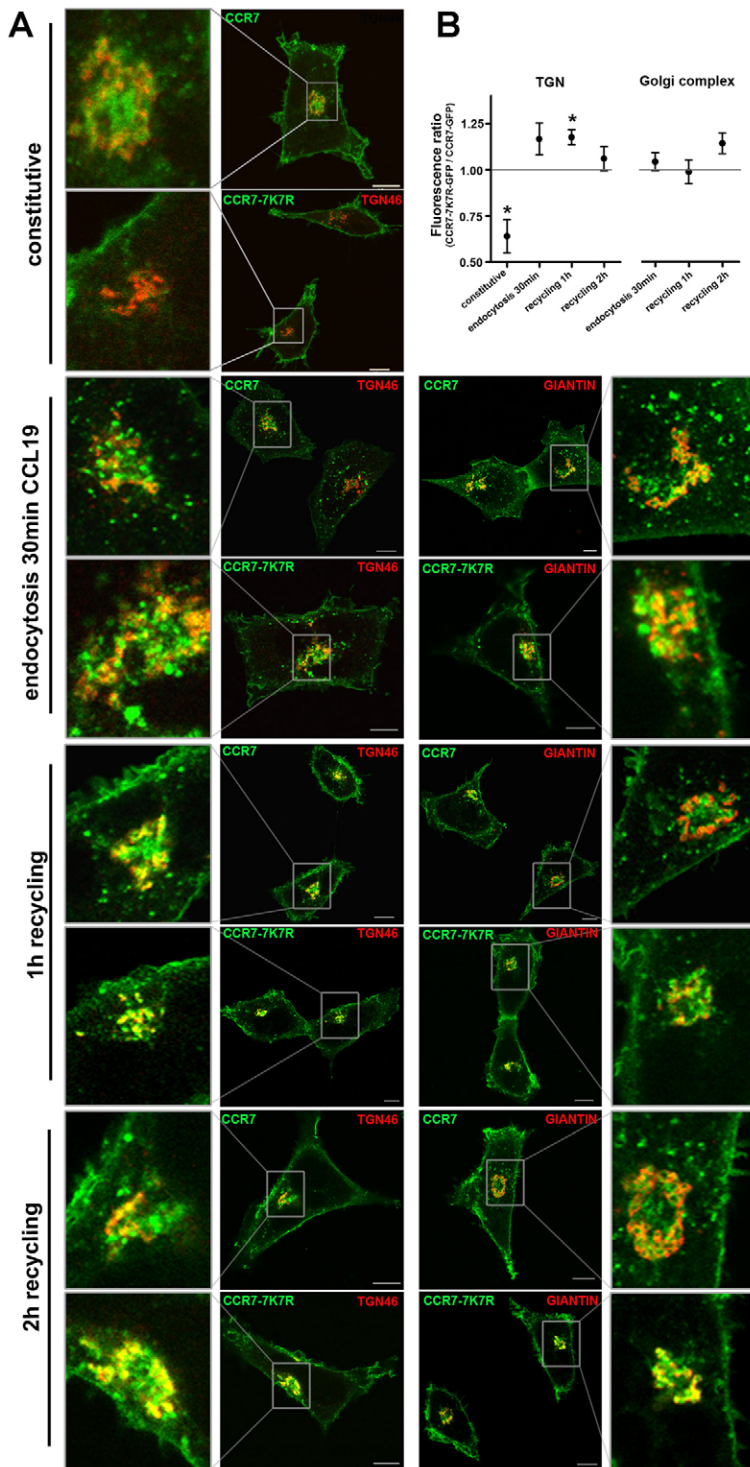
studies at a pH of 5.5 as found in early endosomes. At this reduced pH, CCL19-Fc binding was significantly reduced but there was no difference of CCL19-Fc binding to CCR7 compared to CCR7-7K7R (data not shown). This finding is in line with a previous study showing that CCL19 dissociates from CCR7 in endosomes to be sorted for lysosomal degradation whereas the receptor is recycled (Otero et al., 2006).

#### CCR7 recycles via the trans-Golgi network where its lysineless mutant transiently accumulates

Several putative accumulation sites and hence different trafficking possibilities for transmembrane receptors have been described, including the sorting endosomes, the endosomal

recycling compartment (ERC) and the Golgi apparatus (Borroni et al., 2010; Maxfield and McGraw, 2004). We demonstrated previously that CCR7 colocalized with transferrin receptor, indicating recycling via the ERC (Otero et al., 2006). According to the data depicted in Figs 4 and 5, internalized CCR7 passed the perinuclear region on the way to the plasma membrane. In order to address whether endocytosed CCR7 passes the Golgi compartment on its route back to the plasma

membrane, we carried out a series of colocalization experiments using a GIANTIN-specific antibody as marker for the Golgi apparatus and a TGN46-specific antibody to stain the trans-Golgi compartment. In the absence of chemokines a small fraction of CCR7-GFP was observed at the trans-Golgi network (TGN), whereas the lysineless CCR7 mutant was only marginally detected in this region (Fig. 6). This observation supports a function of receptor ubiquitylation in constitutive receptor



**Fig. 6. CCR7 recycles via the TGN where its lysineless mutant accumulates.** (A) HEK293-CCR7-GFP and CCR7-7K7R-GFP cells were incubated for 30 min with CCL19 to allow receptor internalization and, where indicated, washed to remove residual CCL19 and incubated in medium for 1 to 2 h, facilitating receptor recycling. Cells were fixed and stained with either TGN46 or GIANTIN antibodies. Images are representatives of several cells examined in at least three independent experiments. Scale bars: 10  $\mu$ m. (B) Quantitative analysis of confocal images was performed as described in Fig. 5 and in the Materials and Methods section. A two-tailed Mann-Whitney test was used for statistical analysis.  $*P < 0.05$ .

trafficking. After CCL19 triggering and subsequent recycling, CCR7-GFP partially colocalized with TGN46, indicating that the receptor recycle back to the plasma membrane via the trans-Golgi compartment. Interestingly, CCR7-7K7R-GFP profoundly colocalized with the trans-Golgi marker TGN46 1 h after ligand removal (Fig. 6A) and the intensity of colocalization was significantly higher than for CCR7-GFP (Fig. 6B). Two and 4 hours after ligand deprivation a portion of CCR7-7K7R-GFP still colocalized with TGN46 (Fig. 6; supplementary material Fig. S3D). Furthermore, we observed a slight overlap in the fluorescence of the internalized ubiquitylation-deficient CCR7 mutant and GIANTIN especially at late time points after ligand treatment, implicating that internalized CCR7-7K7R was still present within the Golgi region. Intracellular CCR7-GFP was found in close vicinity of GIANTIN, but did not really localize within the Golgi apparatus (Fig. 6). The reduced number of CCR7-7K7R-containing globular recycling compartments 2 h after ligand treatment additionally strengthen the assumption that ubiquitylation plays a role for the anterograde trafficking of CCR7 away from the trans-Golgi network back towards the plasma membrane.

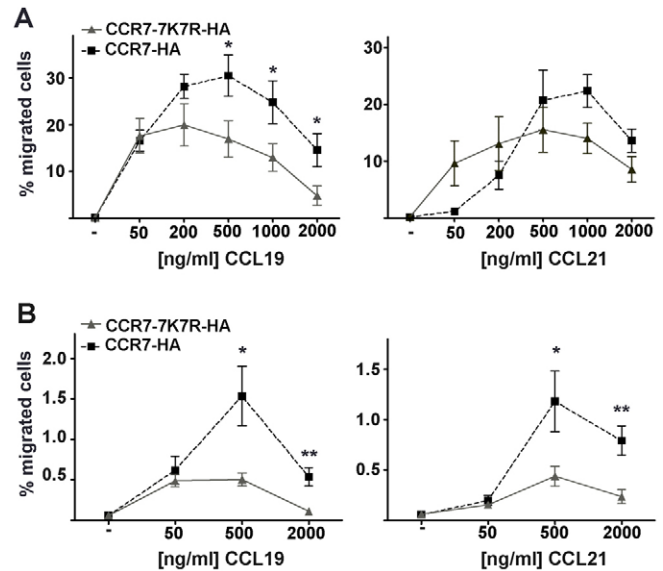
#### Impaired migration of immune cells expressing the lysineless CCR7 mutant

To investigate the impact of receptor ubiquitylation on cell migration, we first performed 2D Transwell™ chemotaxis assays. CCR7-HA expressing 300-19 cells migrated in a dose-dependent manner in response to both chemokines, CCL19 and CCL21, as expected (Fig. 7A). Cells expressing CCR7-7K7R-HA also migrated under these conditions in a dose-dependent manner (Fig. 7A). However, cells expressing CCR7-7K7R migrated significantly less efficient in response to CCL19. Migration towards CCL21 was only diminished at higher chemokine concentration but the reduction was not significant. We next thought to investigate cell migration in a more limiting system, where chemokine sensing must occur for a longer period of time and hence impaired chemokine receptor recycling might show a more severe phenotype. To test this, we investigated chemotaxis of CCR7 transfectants embedded in a 3D collagen matrix. CCR7 expressing cells migrated in response to CCL19 and CCL21 in a 3D environment (Fig. 7B). In contrast, chemotaxis towards both chemokines in 3D of immune cells expressing the lysineless mutant of CCR7 was significantly impaired (Fig. 7B), providing clear evidence that ubiquitylation of CCR7 is pivotal for efficient cell migration in 3D.

In summary, we identified two novel roles for chemokine receptor ubiquitylation, first, in regulating ligand-independent basal receptor trafficking, and second, in the sorting decision of ligand-induced internalized CCR7 within the trans-Golgi compartment, permitting efficient recycling. Moreover, we demonstrated that ubiquitylation of CCR7 plays a key role in immune cell migration.

#### Discussion

Ubiquitylation of transmembrane receptors is known as a signal for receptor internalization and lysosomal sorting. In this study, we identify a novel function for receptor ubiquitylation in the regulation of the trafficking process of the G protein-coupled receptor CCR7. We show that receptor ubiquitylation occurs under basal, ligand-independent conditions. CCR7 ubiquitylation positively affects constitutive receptor trafficking and functions



**Fig. 7. Impaired migration of ubiquitylation-deficient CCR7-expressing cells in a 3D environment.** (A) Stably transfected 300-19 CCR7-HA and CCR7-7K7R-HA cells ( $1 \times 10^5$ ) were allowed to migrate in 2D through Transwell™ filters for 3 h in response to graded concentrations of CCL19 or CCL21. The number of migrating cells was determined by flow cytometry. Mean values  $\pm$  s.e.m. of five independent experiments are depicted as the percentage of input cells. (B) Cell migration in 3D was assessed using 300-19 transfectants embedded in collagen matrix.  $4 \times 10^5$  cells per assay point were allowed to migrate in response to graded concentrations of CCL19 and CCL21 for 3.5 h. Numbers of migrated cells were determined by flow cytometry. Results are the mean values  $\pm$  s.e.m. of at least five independent experiments. To determine the statistical significance a two-tailed, unpaired Student's *t*-test was performed. \* $P < 0.05$ ; \*\* $P < 0.01$ .

as signal for the anterograde trafficking of internalized receptors upon chemokine stimulation. Moreover, we demonstrate that receptor recycling controlled by ubiquitylation plays a vital role in chemokine-mediated immune cell migration.

The pattern of receptor modification by ubiquitin is very diverse. Monoubiquitylation and multiubiquitylation of transmembrane receptors have been reported to control their sorting processes into vesicles at different stages on the endocytic pathway (Haglund et al., 2003; Marchese and Benovic, 2001; Mosesson et al., 2003; Terrell et al., 1998). Polyubiquitylation of receptors predominantly leads to receptor degradation, but also plays a role in internalization and sorting of cargo proteins. Thereby several linkages between ubiquitins are possible (Mukhopadhyay and Riezman, 2007). K48-ubiquitin linkage is mainly used for targeting of the receptor to the degradative, proteasomal pathway, whereas K63-ubiquitin linkage has diverse functions, including in receptor internalization (Terrell et al., 1998; Varghese et al., 2008). Ubiquitylated CCR7 species were apparent at different molecular weights, indicating poly- or multiubiquitylation, but CCR7 seems not to be poly-K48-ubiquitylated (Fig. 1). Of note, CCR7 ubiquitylation was constitutive and occurred also in the absence of ligands. Chemokine triggering of CCR7 only led to a marginal change in the ubiquitylation pattern. Constitutive ubiquitylation was reported for a number of receptors and predominantly serves as signal for endocytosis and/or degradation (Fernández-Sánchez et al., 2009; Gimita et al., 2003). Alternatively, ubiquitylation of



the protease-activated receptor-1 was shown to negatively regulate the constitutive receptor internalization (Wolfe et al., 2007). Moreover, basal ubiquitylation of platelet-activating receptor was reported without determining its role in receptor trafficking (Dupré et al., 2003). Our study suggests that constitutive ubiquitylation of CCR7 has two distinct functions: on the one hand it influences the basal receptor trafficking in a ligand-free environment. On the other hand, ubiquitylation of CCR7 plays a role in receptor recycling after agonist-induced internalization. We identified low but persistent numbers of vesicle-like structures containing CCR7 in the absence of CCL19, which were significantly reduced in the lysineless CCR7 mutant. Most of the constitutively internalized CCR7 localized in early endosomal compartments and were absent in lysosomes.

Until present, ubiquitylation of chemokine receptors was only reported for CXCR4 (Marchese and Benovic, 2001). Chemokine stimulated CXCR4 was ubiquitylated by the E3 ubiquitin ligase AIP4, facilitating trafficking to endosomes where the receptor localized within hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)-positive regions (Marchese et al., 2003). Furthermore the authors suggested that CXCR4 was sorted by the conserved ESCRT (endosomal sorting complex required for transport) machinery. In contrast to CXCR4, we found no evidence for agonist-mediated lysosomal degradation of CCR7 (Otero et al., 2006), neither for CCR7 nor for its lysineless mutant (Fig. 4).

Whereas internalized CXCR4 is degraded, several other chemokine receptors undergo recycling after agonist-mediated internalization (Borroni et al., 2010; Neel et al., 2005). However, studies investigating mechanisms that control the post-endocytic recycling pathways are rare. Commonly, receptor-ligand complexes are internalized by clathrin-dependent or independent mechanisms and subsequently transported to early endosomes, where the mild acidic environment provokes the dissociation of the ligand from the receptor. This early endosomal sorting process is mainly controlled by a distinct subset of Ras-associated binding (Rab) proteins (Grant and Donaldson, 2009). In particular there are two different recycling pathways described – a rapid recycling pathway, directly returning the cargo proteins back to the plasma membrane and a slow recycling path continuing over the endocytic recycling compartment (ERC). Generally, the rapid recycling path is supported by Rab4/Rab5, whereas the slow route is under the control of Rab11 (Borroni et al., 2010; Grant and Donaldson, 2009). CCR7 was previously shown to be internalized in a G protein-independent,  $\beta$ -arrestin-dependent manner via clathrin coated-pits (Comerford et al., 2006; Otero et al., 2008; Otero et al., 2006). In this study we show, that internalized CCR7 recycles via early endosomes through the trans-Golgi compartment to the plasma membrane (Figs 5, 6). Together with a recent report on CCR5 (Escola et al., 2010), we show for the first time that chemokine receptors take the recycling route via the TGN to the plasma membrane.

By studying receptor trafficking using a lysineless CCR7 mutant, we identified a so far unknown role of ubiquitylation as sorting signal for receptor recycling through the TGN. Although we provide clear evidence that CCR7 is directly ubiquitylated at intracellular lysine residue(s) by using a lysineless receptor mutant, we can not fully rule out, that mutating all intracellular lysines might not have additional, so far unidentified defects apart the lack of ubiquitylation. Only a few components are known that

might play a role in this recycling pathway. Rab11 and the Eps15-homology domain protein (EHD1) were shown to regulate the transport from the ERC to the plasma membrane as well as to the TGN (Lin et al., 2001; Wilcke et al., 2000). However, there might be several different routes from the endosomal system to the TGN (Iversen et al., 2001). Components involved in the sorting process at the TGN are not well defined. Recently, GGA proteins were identified to facilitate membrane trafficking at the TGN (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst and Robinson, 1998; Poussu et al., 2000). GGA proteins can directly bind to coat proteins, like clathrin or ARF proteins (Boman, 2001; Puertollano et al., 2001) or can interact with a defined subset of sorting receptors that traffic between TGN and lysosomes (Nielsen et al., 2001; Takatsu et al., 2001). Moreover, a novel aspect of GGA proteins in functioning as ubiquitin sorting receptors at the TGN was reported (Scott et al., 2004). Along this line, a role for ubiquitin in the trafficking of insulin-regulated glucose transporter GLUT4 from the endosomal/TGN system into its intracellular storage compartment was described recently, facilitating the recruitment of GLUT4 to the cell surface upon insulin stimulation (Lamb et al., 2010). Whether members of the GGA protein family are involved in chemokine receptor trafficking is currently not known.

Commonly, chemokine receptor internalization is associated with receptor desensitization resulting in cessation of chemokine signaling (Thelen and Stein, 2008). Interestingly, studies indicated that sequestered receptors are able to transmit signals from intracellular compartments. For instance, blockade of EGFR internalization led to a reduced activity of ERK-1/2 and PI3K (Vieira et al., 1996). Or, attenuated MAPK activation was noted for  $\beta$ 2-adrenergic receptor in cells with impaired  $\beta$ -arrestin-mediated receptor internalization (Daaka et al., 1998). These studies suggest that receptor ubiquitylation might regulate receptor signaling by modulating subcellular CCR7 localization. To explore potential roles of receptor ubiquitylation in signaling, we determined CCR7-dependent activation of ERK-1/2. In fact, we detected a more sustained ERK-1/2 phosphorylation upon chemokine triggering in the ubiquitylation-deficient CCR7 compared to the wild-type receptor (Fig. 2D). Finally, we discovered that chemotaxis was severely impaired in cells expressing the ubiquitylation-deficient CCR7, whereas cells expressing wild-type CCR7 readily migrated in response to CCL19 and CCL21 (Fig. 7). These results suggest that recycling of internalized CCR7, which is hampered in the absence of efficient receptor ubiquitylation, plays a crucial role in directional immune cell migration. This observation is in line with a model predicting that chemokine receptor recycling is necessary to maintain an equilibrated density of receptors at the cell surface permitting cell navigation (Lin and Butcher, 2008) and is supported by a study showing that FIP2 and myosin Vb are required for CXCR2 recycling and chemotaxis (Fan et al., 2004).

In summary, we identified that CCR7 recycles via the TGN and is directly ubiquitylated. We demonstrate that receptor ubiquitylation plays a role for two distinct aspects of CCR7 trafficking. First, CCR7 ubiquitylation enhanced constitutive trafficking in the absence of chemokines. Second, ubiquitylation is involved in the exit of chemokine-internalized CCR7 from the TGN. Moreover, we demonstrate that immune cells expressing this ubiquitylation-deficient CCR7 mutant showed impaired migration.

## Materials and Methods

### Reagents

Human CCL19 and CCL21 were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Antibodies were obtained from the following sources: rat anti-human CCR7 (clone 3D12, BD Biosciences; Allschwil, Switzerland); monoclonal anti-HA-horseradish-peroxidase (HRP) (clone HA7) and anti-Flag-HRP (clone M2) (both from Sigma, Buchs, Switzerland); anti-phospho-p44/42 MAPK (pERK-1/2, Thr202/Tyr204, clone E10) and anti-p44/42 MAPK (ERK-1/2, clone 3A7) (Cell Signaling Technology, Danvers, MA, USA), anti-ubiquitin mAb (clone FK2, Enzo Life Sciences, Lausen, Switzerland), anti-TGN46 (Abcam, Cambridge, UK), anti-EEA1 (BD Biosciences), Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, Basel, Switzerland), Cy3 goat anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK). Streptavidin-phycoerythrin (PE) was from Sigma.

### Cell lines and transfection

Human epithelial kidney 293 (HEK293) cells were grown in DMEM (Lonza, Basel, Switzerland) containing 10% FCS. Murine 300-19 pre-B cells (Legler et al., 1998) were grown in RPMI1640 (Lonza) supplemented with 10% FCS, 50  $\mu$ M  $\beta$ -mercaptoethanol and 2 mM nonessential amino acids. HEK293 cells were transfected by FuGENE6 (Roche, Basel, Switzerland); 300-19 cells by the Amaxa technology. Stable cell lines were generated by a pre-selection step in the presence of 1 mg/ml G418 following a single cell sorting using a FACSArialIIu (BD Biosciences).

### Construction of expression plasmids

Cloning of pcDNA3-CCR7-HA and pcDNA3-CCR7-GFP has been described previously (Otero et al., 2006). The lysineless CCR7-7K7R-HA mutant was generated by site-directed mutagenesis using pcDNA3-CCR7-HA as template. Briefly, to replace the three lysines (K332, K339, K342) located within the C-terminal tail by arginines, a first PCR was conducted with the primer CCR7se2 (5'-ATAGAAATTCCTCATGGACCTGGGGAAAC, restriction site italic) and the primer 3K3Ras (5'-CAGGTCCTGAAGAGCCCTGAAGAGATCGTTGCGGAA-CCTGACGCC, mutated sites underlined). A second PCR was performed using the primers 3K3Rse (5'-GGCGTCAGGTTCCGCAACGATCTTCCAGGCTCTT-CAGGGACCTG, mutated sites underlined) and CCR7as (5'-TATGGCGCCG-TGGGAGAGAAGGTGGTG, restriction site italic). Both PCR products were mixed and a conventional PCR was performed with the primers CCR7se2 and CCR7as, which contain the restriction sites for *EcoRI*/NotI. Lysine residues located in the first (K87, K90) and third intracellular loop (K260, K263) were exchanged to arginines using the following primer pairs: CCR7-7K7Rse1 (5'-TAT-TTCAGGAGGCTCAGGACCATG) and CCR7-7K7Ras1 (5'-CATGGTCTCT-GAGCCTCTGAAATA, mutated sites underlined), and CCR7-7K7Rse2 (5'-CGCAACAGGGCCATCAGGGGTGATC) and CCR7-7K7Ras2 (5'-GATCAC-CCTGATGGCCCTGTTGCG, mutated sites underlined), respectively. Excision of CCR7 from pcDNA3-CCR7-GFP and ligation of CCR7-7K7R into the *EcoRI*/NotI sites resulted in pcDNA3-CCR7-7K7R-GFP. The ubiquitin-mutants 3xFlag-K48R-ubiquitin and 3xFlag-K63R-ubiquitin were generated using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Stratagene, Santa Clara, CA, USA) and the primers K48R (5'-GATCTTTGCCGGTAGGCAGCTCGAGGACG) and K63R (5'-GATTACAACATTTCAGGGGAGTCCGACCTTACATCTTG) (mutated sites underlined). pCMV10-3xFlag-ubiquitin served as template.

### Flow cytometry based receptor endocytosis/recycling assay

CCR7 expressing transfectants were stimulated with 2  $\mu$ g/ml CCL19 for 30 min at 37°C for endocytosis assays. For recycling experiments, cells with internalized receptor were washed twice with warm PBS to remove unbound chemokines and incubated in chemokine-free medium for various time periods at 37°C to allow receptor recycling back to the plasma membrane. Subsequent surface receptor staining was performed at 4°C using a biotinylated CCR7 specific antibody and analyzed by flow cytometry (LSRII, BD Biosciences). Bar graphs were generated using the median value of fluorescence intensity for calculation. Median values of untreated cells served as reference.

### Migration assays

Cell migration in 2D was assessed in 5  $\mu$ m-pore size Transwell™ assays (Corning Costar, Corning, NY, USA).  $1 \times 10^5$  300-19 cells stably expressing either CCR7-HA or CCR7-7K7R-HA were allowed to migrate over 3 h to the lower compartment, which contained various amounts of chemokine. Numbers of migrated cells were assessed by flow cytometry. Number of cells migrating spontaneously without a chemokine trigger, were subtracted.

To determine migration in 3D,  $4 \times 10^5$  of either 300-19 CCR7-HA or CCR7-7K7R-HA cells respectively were resuspended at a 2:1 ratio in a collagen mixture containing PureCol (INAMED Biomaterials, Fremont, CA, USA) with a final collagen concentration of 1.6 mg/ml, 1x MEM (Sigma) and 0.4% sodium bicarbonate (Sigma). The mixture was transferred to 5  $\mu$ m pore size Transwell™ inlets and incubated at 37°C for 40 min to allow collagen polymerization. Filters were transferred into fresh Transwell plates containing

600  $\mu$ l medium supplemented with indicated amounts of chemokines. Cells were allowed to migrate towards the lower, chemokine-bearing compartment for 3.5 h before the number of migrated cells was assessed by flow cytometry.

### Immunoprecipitation

HEK293 cells stably expressing CCR7-HA were transiently transfected with 3xFlag-ubiquitin. Cell lysis was performed 48 h after transfection by incubation at 4°C for 30 min in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 10% glycerol and 0.4% N-dodecyl maltoside). Cell lysates were centrifuged for 20 min at 17,600 g. IgG-agarose precleared lysates were incubated with anti-HA-agarose overnight at 4°C. After extensive washing with lysis buffer, bound proteins were eluted from the anti-HA-agarose with an excess of HA-peptide. The immunoprecipitated proteins in loading buffer containing a final concentration of 1%  $\beta$ -mercaptoethanol were resolved on 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane using a wet blot system. Membranes were blocked for 1 h and incubated with HRP-coupled HA or Flag antibody for 2 h at RT and tagged proteins were detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

### Analysis of chemokine-mediated ERK phosphorylation

$1 \times 10^6$  of HEK293 cells expressing either CCR7-HA or CCR7-7K7R-HA were stimulated with 100 ng/ml CCL19 or CCL21 for different time points and subsequently lysed with NP-40 buffer (50 mM Tris, 1% NP-40, 0.25% sodiumdesoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, pH 7.4) supplemented with a proteinase and phosphatase inhibitor mixture (Roche). Cell lysates were transferred to a 10% SDS-PAGE and western blot analysis was performed using pERK-1/2 and ERK-1/2 antibodies for detection.

### Immunofluorescence confocal microscopy and imaging analysis

Stably transfected CCR7-GFP or CCR7-7K7R-GFP HEK293 cells were plated on coverslips with a density of about 30–40%. Cells were treated with 2  $\mu$ g/ml CCL19 for 30 min at 37°C, washed carefully with cold PBS and fixed with 4% paraformaldehyde. To block reactive aldehyde groups, coverslips were incubated with 50 mM NH<sub>4</sub>Cl for 10 min at 4°C. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at RT. After a further 30 min blocking step with 3% BSA/PBS the microscopic preparations were incubated with the indicated antibodies overnight at 4°C or 1 h at RT. Following intense washing with PBS the secondary antibody was added. Afterwards coverslips were mounted in VECTASHIELD® or polyvinyl alcohol Mounting Media and analyzed by confocal imaging using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany). Images were generated using pinhole size corresponding to 1  $\mu$ m layers for each channel. Contrast and brightness of pictures were simultaneously adjusted with Adobe Photoshop CS2.

Images were analyzed using ImageJ (ImageJ Software version ImageJ 1.45 s). For quantification, first, a mask of the organelle of interest was created using a specific threshold level for the fluorescence in the red channel. This threshold level was identical for each image analyzed for TGN46, GIANTIN or EEA1, respectively. For the TGN (TGN46 staining) and the Golgi complex (GIANTIN staining) areas  $\geq 1 \mu\text{m}^2$  were used to generate the mask. In a second step, mean GFP fluorescence in the specific organelle was determined using the generated mask to specify the area to be measured. Afterwards, the determined mean GFP fluorescence in the corresponding area was normalized to the overall mean GFP fluorescence of the cell. Normalized fluorescent values of either CCR7-GFP or CCR7-7K7R-GFP were used to calculate the fluorescent ratio. For statistical evaluation, fluorescent values of 11–50 cells were used derived from at least two independent experiments.

### Statistical evaluation

Statistical analysis was performed using GraphPad InStat (GraphPad Software version 3.06, Inc., La Jolla, CA). To calculate significances, data sets of at least four independent experiments were used. Individual statistical tests are mentioned in the respective figure legend. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

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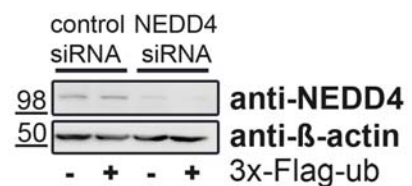
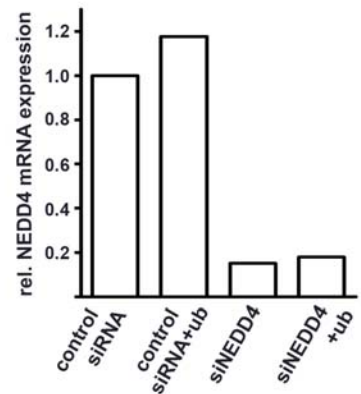
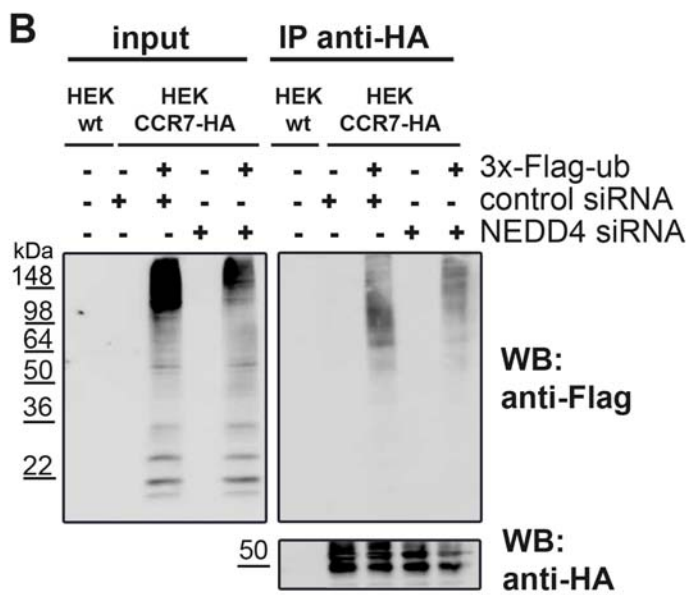
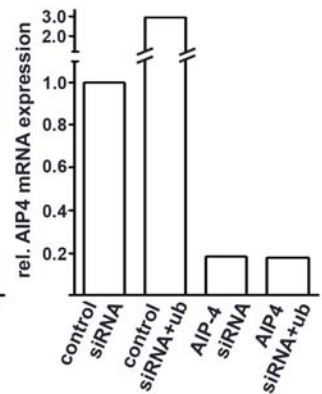
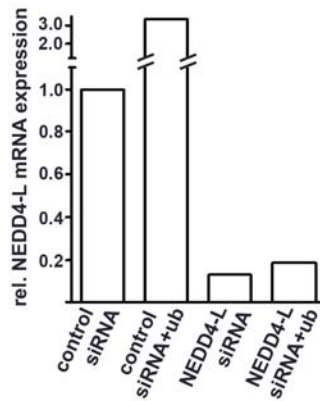
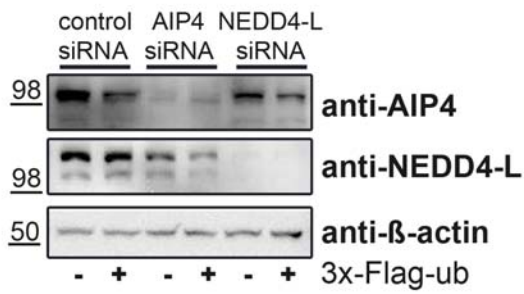
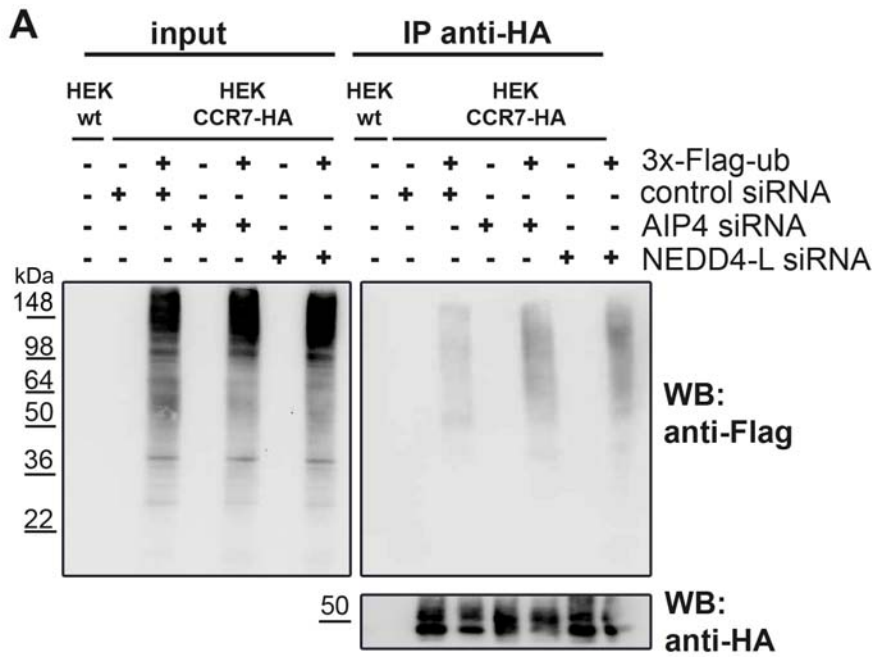
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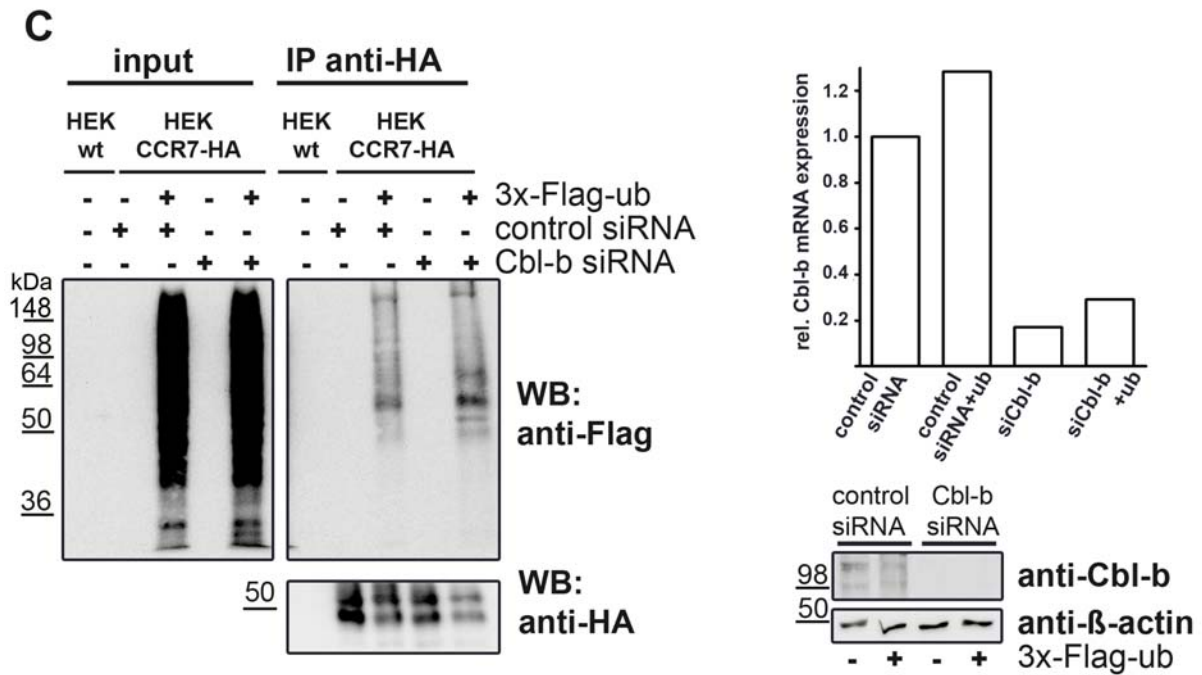
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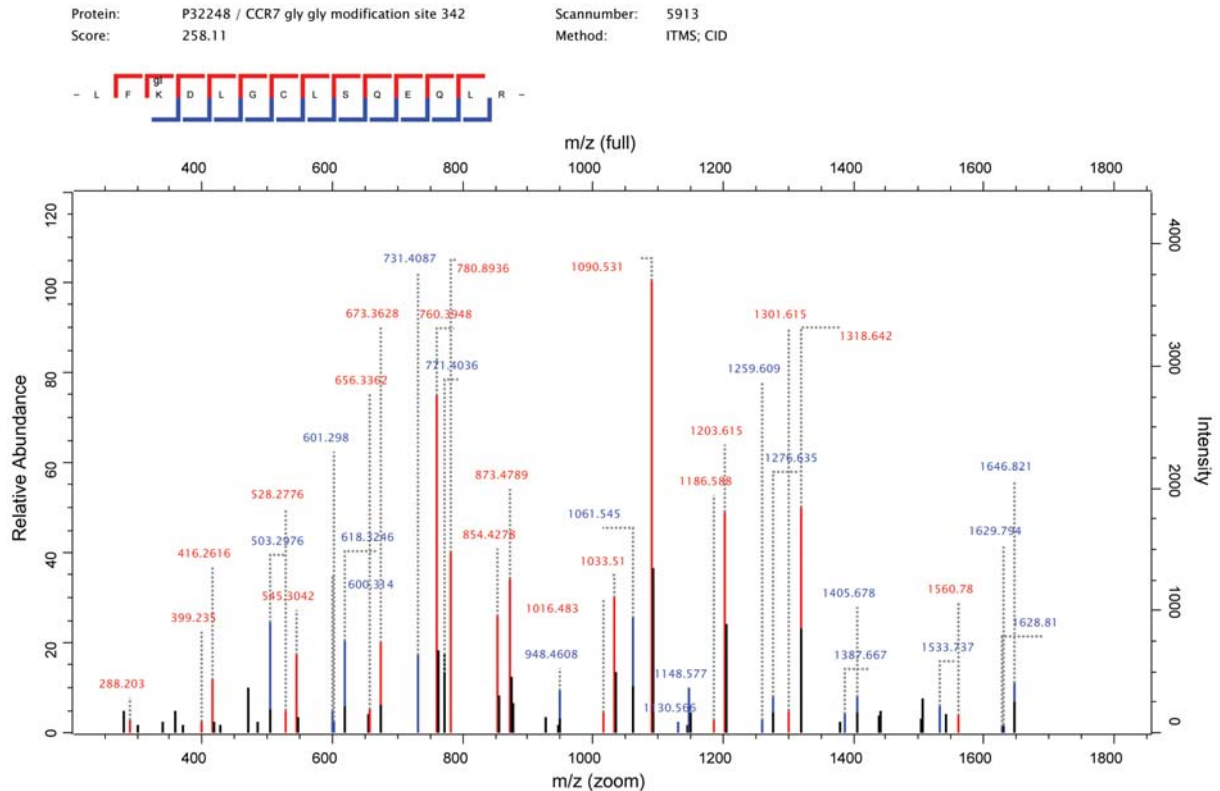
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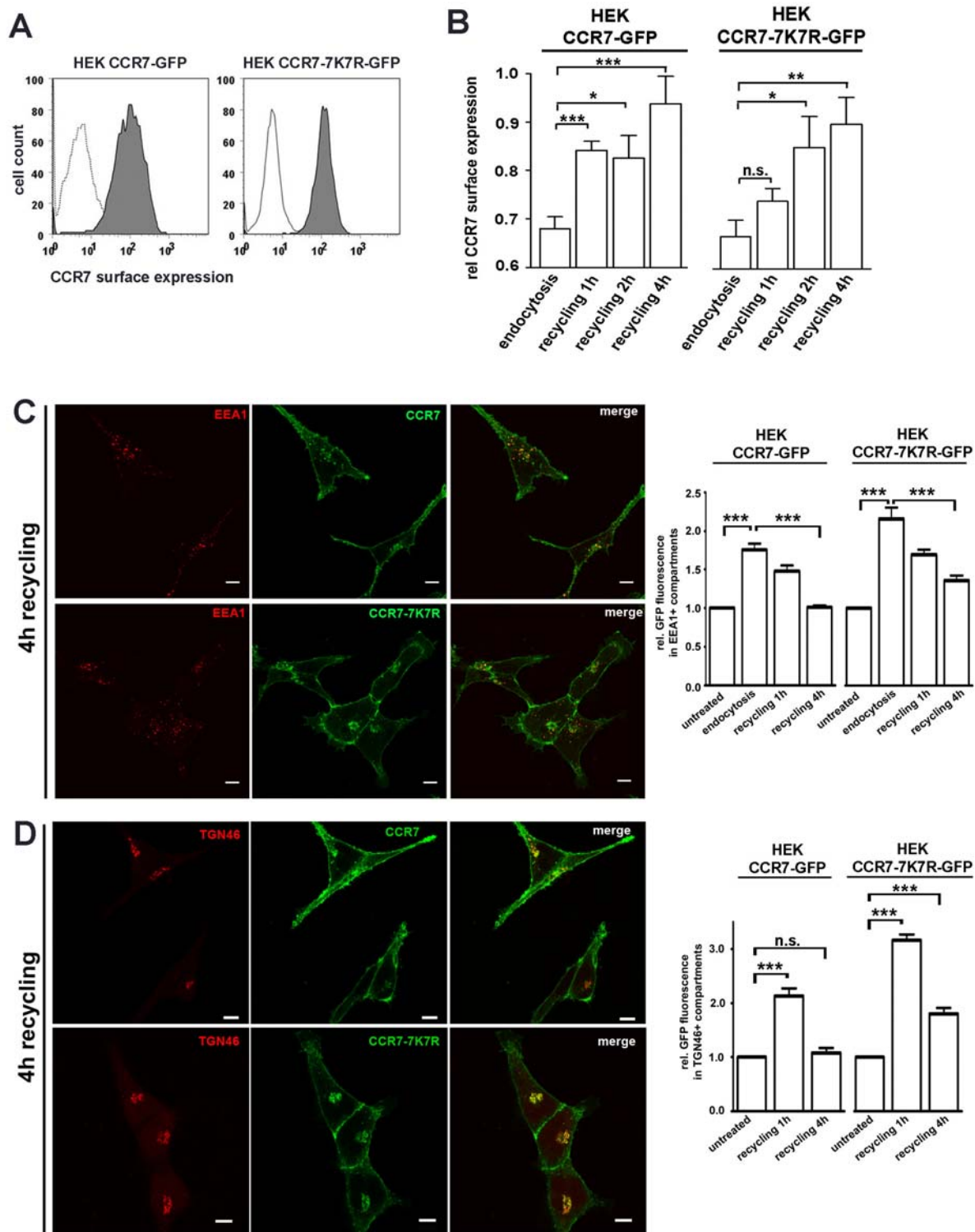
**Fig. S1. The Nedd4 family of E3 ubiquitin ligases and Cbl-b are not responsible for CCR7 ubiquitylation**

HEK293-CCR7-HA cells were transfected using the X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) with siRNA for different E3 ubiquitin ligases, including AIP4 (A), NEDD4-L (A), NEDD4 (B) and Cbl-b (C). For each ligase a mixture of four different siRNAs sequences (Hs\_NEDD4\_3, Hs\_NEDD4\_4, Hs\_NEDD4\_7, Hs\_NEDD4\_8; Hs\_NEDD4L\_3, Hs\_NEDD4L\_5, Hs\_NEDD4L\_6, Hs\_NEDD4L\_10; Hs\_ITCH\_1, Hs\_ITCH\_3, Hs\_ITCH\_5, Hs\_ITCH\_6; Hs\_CBLB\_1, Hs\_CBLB\_2, Hs\_CBLB\_3, Hs\_CBLB\_5; AllStars Negative Control siRNA, Qiagen, Hombrechtikon, Switzerland) was transfected to gain efficient knock-down of the targeted mRNA. Where indicated, cells were additionally transfected with 3xFlag-ubiquitin at day one after siRNA transfection. On day 2, cells were harvested and samples were splitted to either prepare lysates or to isolate mRNA to determine the knock-down efficiency. mRNA was isolated using a RNA isolation kit (RNeasy Mini Kit, Qiagen) following the manufacture's instructions. Next, 1µg mRNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Equal amounts of cDNA were used to perform quantitative real-time-PCR on an Applied Biosystems 7900-HT Fast Real-Time PCR Cycler using Fast SYBRGreen PCR kit (Applied Biosystems) with specific primers for hNEDD4 (Hs\_NEDD4\_1\_SG QuantiTect Primer Assay), hNEDD4-L (Hs\_NEDD4L\_1\_SG QuantiTect Primer Assay), hAIP-4 (Hs\_ITCH\_1\_SG QuantiTect Primer Assay) or hCbl-b (Hs\_CBLB\_1\_SG QuantiTect Primer Assay). Expression levels were normalized to the housekeeping gene GAPDH (primers Hs\_99999905\_m1). Residual cells were lysed and CCR7 was immunoprecipitated, using anti-HA-agarose. Ubiquitylation of the receptor was determined by immunoblotting with the HRP-coupled anti-Flag antibody M2. To control the knock-down efficiency on protein level, lysates were additionally immunoblotted to detect the respective ligase. The same blots were striped and β-actin was detected to assure equal protein loading.



### Fig. S2. Identification of direct ubiquitylation of CCR7 by mass spectrometry

HEK293 cells expressing strep-tagged CCR7 were lysed in the presence of protease inhibitor and iodoacetamide. Subsequently, a two-step purification was performed, first a strep affinity purification, followed by an anti-ubiquitin (clone FK2) affinity purification. Eluates were digested with trypsin and analysed by mass spectrometry using a LTQ Orbitrap XL. The raw data was analysed with Maxquant as described (Cox, J. and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26**, 1367-72). An ubiquitylation site (di-glycine modification) was identified on lysine 342 of CCR7 with high confidence in multiple fragmentation spectra of three replicas (precursor charge 3+, mass: 607.64897 m/z, Mass Error [ppm] -0.43121, Maxquant score 258.11).



**Fig. S3. Retarded recycling of internalised CCR7-7K7R-GFP**

(A) Comparable cell surface receptor expression of CCR7-GFP and CCR7-7K7R-GFP in stably transfected HEK293 cells was assessed by flow cytometry. (B) Ligand-mediated endocytosis and recycling of CCR7 was determined in cells stimulated with  $2\mu\text{g/ml}$  CCL19 for 30 min. For receptor recycling, CCL19-stimulated cells were washed extensively to remove unbound ligand and further incubated for 1 to 4h in the absence of chemokine. Cells were subsequently put on ice and the surface expression levels of CCR7 were determined by flow cytometry. Bar graphs indicated the result of five (2 & 4h recycling) to twelve



independent experiments. (C,D) Stably transfected HEK293-CCR7-GFP and CCR7-7K7R-GFP cells were incubated for 30 min with CCL19, washed extensively to remove unbound chemokine and incubated in media for 4 h. Cells were fixed and stained for either EEA1 (C) or TGN46 (D). Images are representatives of several cells examined in at least two independent experiments. Scale bars are 10  $\mu$ m. For quantification, mean GFP fluorescence in EEA1- or TGN46-positive compartments, respectively, was determined and normalized to the overall mean GFP fluorescent of the cell as described in the method section. The values derived from CCR7-7K7R-GFP and CCR7-GFP expressing cells from the indicated stimulations were normalized to the GFP value of the untreated (constitutive) control value of the corresponding cell type and depicted as relative GFP fluorescence. For statistical analysis (B-D), data were subjected to a One-way ANOVA with Bonferroni multiple comparison post test. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s. not significant.