Klebsiella pneumoniae targets an EGF receptor-dependent pathway to subvert inflammation

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Summary

The NF-κB transcriptional factor plays a key role governing the activation of immune responses. Klebsiella pneumoniae is an important cause of community-acquired and nosocomial pneumonia. Evidence indicates that K. pneumoniae infections are characterized by lacking an early inflammatory response. Recently, we have demonstrated that Klebsiella antagonizes the activation of NF-κB via the deubiquitinase CYLD. In this work, by applying a high-throughput siRNA gain-of-function screen interrogating the human kinome, we identified 17 kinases that when targeted by siRNA restored IL-1β-dependent NF-κB translocation in infected cells. Further characterization revealed that K. pneumoniae activates an EGF receptor (EGFR)-phosphatidylinositol 3-OH kinase (PI3K)–AKT–PAK4–ERK–GSK3β signalling pathway to attenuate the cytokine-dependent nuclear translocation of NF-κB. Our data also revealed that CYLD is a downstream effector of K. pneumoniae-induced EGFR–PI3K–AKT–PAK4–ERK–GSK3β signalling pathway. Our efforts to identify the bacterial factor(s) responsible for EGFR activation demonstrate that a capsule (CPS) mutant did not activate EGFR hence suggesting that CPS could mediate the activation of EGFR. Supporting this notion, purified CPS did activate EGFR as well as the EGFR-dependent PI3K–AKT–PAK4–ERK–GSK3β signalling pathway. CPS-mediated EGFR activation was dependent on a TLR4–MyD88–c-SRC-dependent pathway. Several promising drugs have been developed to antagonize this cascade. We propose that agents targeting this signalling pathway might provide selective alternatives for the management of K. pneumoniae pneumonias.

Introduction

Activation of innate responses is a key event in the host defence against pathogens. These responses rely on the activation of signalling pathways resulting in the production of antimicrobial molecules, the expression of co-stimulatory molecules and the release of cytokines and chemokines (Mogensen, 2009). These signalling pathways converge on a limited set of transcriptional activators including NF-κB, mitogen-activated protein kinases (MAPKs) and IFN regulatory factor(s) (IRF) controlling the transcription of genes coding for a variety of inflammatory mediators (Medzhitov, 2007; Kumar et al., 2009; Mogensen, 2009). Evidence supports the notion that infections share a common host response, the so-called ‘alarm signal’, under the control of a NF-κB-dependent signalling pathway upon recognition of the pathogen by germ line-encoded receptors referred to as pattern recognition receptors (PRRs) (Jenner and Young, 2005). For many pathogens, activation of this fast-acting host response leads to clearance of the infection. Not surprisingly, subversion of this response is considered important for pathogen survival. A common strategy share by many pathogens is to block the activation of the NF-κB-dependent signalling pathway (Rahman and McFadden, 2011; Le Negrate, 2012). Nonetheless, for most of the pathogens there is paucity of information on the cellular determinants targeted to prevent the activation of this signalling pathway.

The NF-κB signalling pathway has been extensively investigated. Functional NF-κB is assembled through homo- or heterodimerization of the five subunits: p65

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(RelA), RelB, c-Rel, p105/p50 (nfkb1) and p100/p52 (nfkb2) being the p65:p50 dimer the primary mediator of inflammation (Hayden and Ghosh, 2008). Under resting conditions, NF-κB dimers reside in the cytoplasm sequestered by members of the IκB family of inhibitory proteins. However, the stimulation of a suitable signalling cascade results in the phosphorylation, K48-linked polyubiquitylation and the subsequent degradation of the inhibitory protein by the proteasome hence leading to the nuclear translocation of NF-κB. When this occurs it triggers the transcription of target genes (Hayden and Ghosh, 2008).

*Klebsiella pneumoniae* is a Gram-negative pathogen which causes a wide range of infections, from urinary tract infections to pneumonia, the latter being particularly devastating among immunocompromised patients with mortality rates between 25% and 60% (Sahly and Podschun, 1997). In addition, *K. pneumoniae* is one of the most frequent antibiotic-resistant bacteria isolated in hospitals but also in the community (Nordmann *et al*., 2009). The best characterized virulence factor of this pathogen is the capsule polysaccharide (CPS). Isogenic CPS mutant strains are avirulent not being able to cause pneumonia and urinary tract infections (Camprubi *et al*., 1993; Cortes *et al*., 2002; Lawlor *et al*., 2005).

A wealth of evidence indicates that ‘Toll-like’ receptor (TLR)-controlled inflammatory responses are essential to clear *K. pneumoniae* infections (Greenberger *et al*., 1996; Stallord *et al*., 1999; Ye *et al*., 2001; Schurr *et al*., 2005; Cai *et al*., 2009). Conversely, this suggests that *K. pneumoniae* tries to counteract the induction of these host defence responses. Indeed, we have shown that, in sharp contrast to wild-type strains, avirulent CPS mutants activate an inflammatory programme through TLR-dependent pathways (Regueiro *et al*., 2006; Moranta *et al*., 2010). Therefore, a key step in *K. pneumoniae* pathogenesis is its ability to modulate the innate immune system in its own benefit. Recently, we have demonstrated that *K. pneumoniae* dampens the activation of the NF-κB pathway, which plays a major role in turning on the inflammatory response upon *K. pneumoniae* infection (Regueiro *et al*., 2011). Thus, *K. pneumoniae* inhibits the cytokine-dependent nuclear translocation of NF-κB by affecting the K63-linked ubiquitination status of key intermediates of the signalling pathway in a process dependent on the activation of the deubiquitinase CYLD (Regueiro *et al*., 2011). This anti-inflammatory phenotype is dependent on alive bacteria–cell contact and *K. pneumoniae* CPS plays a necessary role although it is not the only bacterial factor involved (Regueiro *et al*., 2011).

The present study was designed to identify additional cellular determinants targeted by *K. pneumoniae* to prevent the activation of the NF-κB signalling pathway. To take an unbiased systematic approach towards the identification of these cellular factors, we performed a high-throughput siRNA-mediated screen targeting the human kinome. Using this approach, we identified 17 kinases that when targeted by siRNA restored IL-1β-dependent NF-κB translocation in infected cells. Further characterization revealed that *K. pneumoniae* activates an EGF receptor (EGFR)-phosphatidylinositol 3-OH kinase (PI3K)–AKT–PAK4–ERK–GSK3β signalling pathway to attenuate the cytokine-dependent nuclear translocation of NF-κB.

**Results**

*High-throughput siRNA screening*

We developed an assay suitable for a siRNA-based high-throughput screening for the identification of host kinases involved in the anti-inflammatory effect elicited by *K. pneumoniae* 52145 (hereafter Kp52145). We took advantage of the recently described SIB01 cell line (Bartfeld *et al*., 2010), a derivative of the human alveolar epithelial cell line A549 that expresses an additional copy of the NF-κB p56 subunit fused to green fluorescent protein (GFP). This allowed for detection of p65 translocation to the nucleus by automated fluorescence microscopy. A 3 h infection of SIB01 with Kp52145 resulted in a complete block of IL-1β-induced p65–GFP translocation (Fig. 1A). Similar findings had been obtained previously when non-transduced A549 cells were infected with Kp52145 and p65 was detected by immunofluorescence microscopy (Regueiro *et al*., 2011).

The assay was then transferred from a 24-well format to a 96-well format by optimizing culture conditions, cell number per well, multiplicity of infection, time of contact, amount of siRNA, and stimulation dose and length of IL-1β exposure. The screening workflow is depicted in Fig. 1B and the conditions are described in Experimental procedures. An assay quality Z-score of 0.8 was achieved, indicating that the assay was robust enough for a siRNA-based high-throughput screening (Birmingham *et al*., 2009; Mazur and Kozak, 2012).

The image analysis algorithm used for the analysis of recorded images recognizes cells based on the shape and size of objects (nuclei) in the DAPI channel, and then sorts them into three categories (gates) based on the ratio of the GFP signal intensity in the nucleus area (GFPnuc) versus the GFP signal intensity in the surrounding area (pixels) (cytoplasm, GFPcyt) (Fig. S1). Present in the “high” gate were cells with a high GFPnuc versus GFPcyt ratio which indicates the nuclear translocation of p65 in these cells. The “low” gate contained cells with a low GFPnuc to GFPcyt, indicative of no or very little p65 translocation to the nucleus. The “int” gate represents a narrower threshold region with intermediate GFPnuc to GFPcyt ratios. Control experiments showed that
treatment of transfected cells with AllStars siRNA with the agonist IL-1β (30 min, 10 ng ml⁻¹) placed more than 90% of the cell population in the 'high' gate. In contrast, only 5% of the infected cells were found in this gate and just a mere 8% of the cells infected and treated with IL-1β were gated as positive for p65 translocation.

To screen the human kinome for kinases that when silenced lead to loss of the Kp52145-induced block of IL-1β-dependent p65 nuclear translocation, we used a siRNA library that contained 646 individual pools distributed over 96-well microtitre plates, each well containing two siRNAs targeting the same kinase (Fig. 1C). Three independent rounds of screening were performed and for the analysis of the results the standard Z-score normalization procedure was used (see Experimental procedures) (Birmingham et al., 2009). Figure 1D shows the distribution of mean Z-scores for the three screening rounds considering only the cell population in the ‘high’ gate. The data were rank by the mean Z-score and candidates identified as those with Z-score deviations of > 1.5 were selected. Using this cut-off, we identified 31 gene candidates that when targeted by siRNA produced a relief on Kp52145-imposed block on IL-1β-dependent p65 nuclear translocation (Table S1). We also selected four additional candidates that narrowly did not match this criterion (Table S1). Altogether, we selected 35 gene candidates for independent validation since siRNA-based...
screens are potentially prone to yield/identify false positive hits due to ‘off-target’ effects (Birmingham et al., 2009).

To validate this candidate set, four new siRNAs for each of the 35 selected targets were analysed individually in three independent screening rounds. We considered validated gene candidates only those in which at least two of four siRNAs had a mean Z-score > 1.5. Using this validation criterion, 17 gene candidates were validated, and six of them were considered high-confidence hits (at least three of four siRNAs had a mean Z-score > 1.5; ILK, PAK4, CDK9, HGS, PKMYT1 and PRKACB) (Table 1). Additionally, we asked whether any of the validated siRNAs affect the basal levels of p65 translocation in non-treated cells and/or increase the levels of p65 translocation in Kp52145-infected cells. This was not the case for any of the siRNAs tested (data not shown).

In summary, our high-throughput siRNA-mediated screen targeting the human kinome led to the identification of 17 host kinases that when targeted by siRNA attenuated Kp52145-induced anti-inflammatory effect. For further in-depth analysis, we selected the most potent siRNA for each of the six high-confidence hits with the exception of ILK, where the most potent siRNA also showed considerable cell toxicity.

**Table 1.** Validated targets (Z-score cut-off 1.5).

| Symbol | Gene ID | Function | # of positive siRNAs |
|--------|---------|----------|----------------------|
| ILK    | 3611    | Integrin-linked kinase | 4 of 4 |
| PAK4   | 10298   | p21(CDK1)-activated kinase | 4 of 4 |
| CDK9   | 1025    | Cyclin-dependent kinase 9 (CDC2-related kinase) | 3 of 4 |
| HGS    | 9146    | Hepatocyte growth factor-regulated tyrosine kinase substrate | 3 of 4 |
| PRKACB | 5567    | Protein kinase, CAMP-dependent, catalytic, beta | 3 of 4 |
| PKMYT1 | 9088    | Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase | 3 of 4 |
| CDK5   | 1020    | Cyclin-dependent kinase 5 | 2 of 4 |
| CIB3   | 117286  | Calcium and integrin binding family member 3 | 2 of 4 |
| DAPK2  | 23604   | Death-associated protein kinase 2 | 2 of 4 |
| HIPK1  | 204851  | Homeodomain interacting protein kinase 1 | 2 of 4 |
| MK-STYX| 51657   | Map kinase phosphatase-like protein MK-STYX | 2 of 4 |
| PCTK3  | 5129    | PCTAIRE protein kinase 3 | 2 of 4 |
| PIK3R1 | 5295    | Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) | 2 of 4 |
| PNKP   | 11284   | Polynucleotide kinase 3-phosphatase | 2 of 4 |
| SRPK1  | 6732    | SFRS protein kinase 1 | 2 of 4 |
| TYRO3  | 7301    | TYRO3 protein tyrosine kinase | 2 of 4 |
| UCK1   | 83549   | Uridine–cytidine kinase 1 | 2 of 4 |

**Analysis of the high-confidence validated kinases**

In the canonical NF-κB activation pathway, nuclear translocation of NF-κB is preceded by phosphorylation and subsequent degradation of IκBα (Hayden and Ghosh, 2008). We analysed the levels of IκBα in siRNA-transfected cells by immunoblot. Consistent with our previous results (Regueiro et al., 2011), in cells transfected with control siRNA, IL-1β induced a complete degradation of IκBα already after 10 min whereas infection with Kp52145 abrogated the IL-1β-induced degradation of IκBα (Fig. 2). In contrast, in those cells transfected with siRNAs for each of the six high-confidence hits we did not observe the stabilization of IκBα levels in Kp52145-infected cells after IL-1β challenge (Fig. 2). Collectively, these results indicate that the kinases studied are involved in Kp52145 attenuation of IL-1β-triggered activation of the NF-κB canonical pathway.

To predict functional interactions of the kinases found in the screening, the STRING database (Szklarczyk et al., 2011) was interrogated with the 17 validated hits (Fig. 3). This bioinformatics analysis revealed a clear interconnected group of hits around EGFR, PI3K, AKT and ERK whereas no clear potential interaction partners were identified for nine of the hits (TYRO3, PNK, DAPK2, SRPK1, PKMYT1, CDK9, UCK1, HIPK1, MK-STYXL1) (Fig. 3A). The possibility that EGFR, PI3K, AKT and ERK may act as central nodes of a pathway targeted by Kp52145 to prevent the activation of the NF-κB signalling pathway was further highlighted when the six high-confidence hits were used to interrogate the STRING database (Fig. 3B). We then investigated the functional significance of this *in silico* identified network on *K. pneumoniae*-triggered anti-inflammation.

**Effect of K. pneumoniae infection on phosphorylation of PAK4, ERK, AKT and GSK3β**

We first assessed whether PAK4, one of the high-confidence hits, becomes activated during infection with Kp52145. Immunoblot analysis demonstrated that Kp52145 infection of A549 cells induced the phosphorylation of PAK4 (Fig. 4A). The levels peaked already after 15 min post infection and were sustained till 120 min post
infection. PAK4 has been shown to act upstream of several signalling pathways including ERK-dependent ones (Li and Minden, 2005). Further confirming previous findings (Wu et al., 2006; Regueiro et al., 2008), Kp52145 induced the phosphorylation of ERK (Fig. 4B). Therefore, we sought to determine whether Kp52145-induced ERK phosphorylation is dependent on PAK4. Kp52145-triggered ERK phosphorylation was reduced in PAK4 knockdown cells by siRNA hence suggesting that indeed PAK4 acts upstream of ERK in Kp52145-infected cells (Fig. 4C). We then examined the effect of ERK on Kp52145 anti-inflammation. We silenced expression of ERK1 and ERK2 in A549 cells using a siRNA that targets both, and analysed IL-1β-dependent IκBα degradation in infected cells (Fig. 4D). Kp52145 did not longer attenuate IL-1β-induced IκBα degradation in ERK knockdown cells. Altogether, these results give initial experimental support to the STRING analysis and suggest that Kp52145 does target PAK4–ERK to block NF-κB activation.

Giving the central role of ERK in cell signalling we sought to determine whether Kp52145-induced ERK phosphorylation is affected in cells knockdown for any of the other high-confidence hits. However, likewise in cells transfected with control siRNA, Kp52145 still induced the phosphorylation of ERK in cells knockdown for CDK9, HGS, PKMYT1, PRKACB or ILK (Fig. S2).

The bioinformatics analysis also indicated that PI3K and AKT may be nodes of the pathway targeted by Kp52145. Indirectly supporting this, PIK3R1, the p85 regulatory subunit of PI3K, was one of the validated hits and AKT is a known kinase downstream of PI3K (Wu et al., 2006; Franke, 2008). Immunoblot analysis confirmed that Kp52145 caused a marked increase in AKT phosphorylation which was detected already at 15 min post infection (Fig. 5A). Kp52145-triggered AKT phosphorylation was PI3K-dependent since AKT phosphorylation was not detected in Kp52145-infected cells treated with the PI3K inhibitor LY294002 (Fig. 5B). Furthermore, PAK4 and ERK are downstream of PI3K since treatment of cells with LY294002 inhibited the infection-dependent phosphorylation of both kinases (Fig. 5B). To further sustain that indeed Kp52145 targets PI3K–AKT to prevent NF-κB activation we asked whether treatment of cells with LY294002 attenuates Kp52145-induced anti-inflammatory effect. Results shown in Fig. 5C demonstrated that this was the case.

GSK3β, identified as a node in the bioinformatics analysis, is a constitutively active kinase under resting conditions, but becomes inactivated by phosphorylation of serine 9 by AKT (Cross et al., 1995; Wang et al., 2011). Since Kp52145 activated the phosphorylation of AKT we reasoned that GSK3β may become inactivated upon Kp52145 infection in a PI3K–AKT-dependent manner. Indeed, Kp52145 triggered the phosphorylation of GSK3β, which was abolished in infected cells treated with LY294002 (Fig. 6A). Pretreatment of cells with
pharmacologic mitogen-activated protein kinase (MEK) inhibitor also blocked Kp52145-triggered GSK3β phosphorylation thereby suggesting that ERK participates in Kp52145-induced phosphorylation of GSK3β and, furthermore, that ERK is located upstream of GSK3 (Fig. 6B). As expected, pretreatment of cells with the AKT inhibitor (AKT X) abrogated Kp52145-triggered phosphorylation of ERK and GSK3β (Fig. S3).

To examine the role of inhibitory phosphorylation of GSK3β on Kp52145 anti-inflammatory phenotype, we studied the effect of forced expression of GSK3β. Vectors encoding HA tag wild-type GSK3β (GSK3β-WT) or a
constitutive active mutant (GSK3β-CA) in which the serine 9 residue was changed to alanine were transiently trans-
ected to A549 cells. As control, cells were transfected 
with the empty vector pcDNA3. The Kp52145-induced 
block of IL-1β-triggered IκBα degradation was evident in 
cells transfected with the empty vector (Fig. 6C). In con-
trast, the suppressive effect of Kp52145 was partially 
inhibited in cells transfected with the GSK3β-WT vector 
and abolished in cells transfected with the GSK3β-CA 
vector (Fig. 6C). Therefore, these data suggest that the
inhibitory phosphorylation of GSK3β is required for Kp52145 attenuation of IL-1β-triggered activation of the NF-κB pathway.

In summary, the evidence presented supports the notion that Kp52145 activates a PI3K–AKT–PAK4–ERK–GSK3β-dependent signalling pathway to prevent NF-κB activation.

CYLD is a downstream effector of K. pneumoniae-induced PI3K–AKT–PAK4–ERK–GSK3β signalling pathway

We considered the possibility that the identified pathway is required for the expression of a negative regulator(s) of the NF-κB signalling pathway. We have recently reported that Kp52145 increases the levels of cyl/d mRNA and CYLD protein levels to downregulate the NF-κB pathway (Regueiro et al., 2011). CYLD is a deubiquitinase that removes K63-linked polyubiquitin chains from members of the TRAF family thereby acting as a negative regulator of NF-κB activation (Trompouki et al., 2003; Sun, 2008). We investigated whether Kp52145-induced CYLD expression is dependent on the activation of the PI3K–AKT–PAK4–ERK–GSK3β signalling pathway. We utilized pharmacologic inhibitors of PI3K (LY294002), AKT (AKT X) or MEK (U0126) to block the pathway at three different levels. CYLD protein levels were determined by Western blot analysis. Results shown in Fig. 7A revealed that Kp52145-induced CYLD expression was reduced in those cells pretreated with the inhibitors. Likewise, Kp52145 did not increase the expression of CYLD in cells transfected with GSK3β-CA vector (Fig. 7B).

In our previous study we demonstrated the effect of CYLD on distal elements, TRAF6, of the NF-κB signalling pathway (Regueiro et al., 2011). To test how CYLD affects the Kp52145-dependent block of IL-1β-induced IκBα degradation we silenced CYLD expression using siRNA. In CYLD knockdown cells, Kp52145 did not longer block IL-1β-triggered degradation of IκBα (Fig. 7C). Notably, Kp52145 infection induced the degradation of IκBα in CYLD knockdown cells in sharp contrast to cells transfected with control siRNA (Fig. 7C). Further confirming this result, time course experiments revealed that Kp52145 did induce the degradation of IκBα in CYLD

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knockdown cells already 60 min post infection (Fig. 7D). In good agreement, Kp52145 triggered the nuclear translocation of the NF-kB p65 subunit in CYLD knockdown cells (Fig. 7E).

Previously we have shown that TLR2 and TLR4 play a major role in detecting K. pneumoniae mutants activating NF-kB-dependent responses (Moranta et al., 2010; March et al., 2011). Furthermore, our data revealed that the activation of these responses was dependent on the adaptor molecule MyD88 (Moranta et al., 2010; March et al., 2011). Therefore, we asked whether the increased degradation of IxBα found in Kp52145-infected CYLD knockdown cells is dependent on the engagement of a TLR–MyD88 signalling pathway. Kp52145 did not induce the degradation of IxBα in cells double knockdown for CYLD and MyD88 (Fig. 7F). As expected, IxBα degradation was not observed in MyD88 single knockdown infected cells (Fig. 7F). We next assessed the involvement of TLR2 or TLR4 on Kp52145-induced degradation of IxBα in cells knockdown for CYLD. Kp52145 still triggered the degradation of IxBα in cells double knockdown for CYLD and either TLR2 or TLR4 (Fig. S4) whereas this was not the case in cells triple knockdown for CYLD, TLR2 and TLR4 (Fig. 7G).

Altogether, our data suggest that the activation of the PI3K–AKT–PAK4–ERK–GSK3β signalling pathway by Kp52145 is required to increase the expression of CYLD. Our data further highlight the critical role of CYLD to attenuate TLR signalling cascades upon Klebsiella infection.

Klebsiella pneumoniae-induced anti-inflammatory effect also relies on the activation of EGF receptor

In a recent work we have shown that Kp52145 engages NOD1 to exert its anti-inflammatory effect (Regueiro et al., 2011). Further, activation of NOD1 also mediates Kp52145-induced CYLD expression (Regueiro et al., 2011). Therefore we investigated the potential contribution of NOD1 to Kp52145-triggered activation of PI3K–AKT–PAK4–ERK–GSK3β signalling pathway. To analyse the activation of the pathway we used as cellular readout the detection of the phosphorylations of AKT and ERK upon infection. In NOD1 knockdown cells, Kp52145 was still able to induce the phosphorylation of AKT with similar kinetics and to similar levels to those in cells transfected with control siRNA (Fig. 8A). In turn, although Kp52145-induced ERK phosphorylation was still detected in NOD1 knockdown cells the levels were lower than those found in infected cells transfected with control siRNA (Fig. 8A). Altogether, these data suggest that there might be an additional receptor(s) contributing to the activation of the PI3K–AKT–PAK4–ERK–GSK3β signalling pathway.

Since the bioinformatics analysis indicated that Kp52145 may target EGFR and there is ample evidence demonstrating that EGFR activates PI3K–AKT and ERK-dependent signalling pathways (Henson and Gibson, 2006), we sought to determine whether EGFR could be the receptor involved. First, we investigated whether Kp52145 triggers the phosphorylation of EGFR. Indeed, Kp52145 infection induced the phosphorylation of EGFR (Fig. 8B).

Second, we asked whether Kp52145 still blocks IL-1β-dependent IxBα degradation in EGFR knockdown cells. Results displayed in Fig. 8C show that Kp52145 did not longer block IL-1β-triggered degradation of IxBα in EGFR knockdown cells. Interestingly, in cells double knockdown for EGFR and NOD1 we observed a more important relief of Kp52145-induced block on cytokine-triggered IxBα degradation effect than in the single

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knockdown cells (Fig. 8C). Third, we questioned whether Kp52145 activates the PI3K–AKT–PAK4–ERK–GSK3β signalling pathway via EGFR. Pretreatment of cells with the pharmacologic EGFR inhibitor AG1478 reduced Kp52145-dependent phosphorylation of AKT, ERK and GSK3β hence placing EGFR upstream of the signalling pathway (Fig. 8D). Furthermore, EGFR inhibitor AG1478 reduced Kp52145-induced CYLD expression (Fig. 8E).

Overall, these findings support the notion that Kp52145 may engage EGFR to exert its anti-inflammatory effect.

**Activation of EGF receptor is dependent on K. pneumoniae capsule polysaccharide**

We sought to identify the bacterial factor(s) implicated in the activation of EGFR. Given the importance of bacterial surface molecules on host–pathogen interactions, we...
Fig. 8. *Klebsiella pneumoniae*-induced anti-inflammatory effect relies on the activation of EGFR.
A. Immunoblot analysis of P-Akt, P-ERK and tubulin levels in lysates of siRNA-transfected (AS-, AllStars control siRNA; NOD1 siRNA) A549 cells infected with Kp52145 for the indicated times.
B. Immunoblot analysis of phosphorylated EGFR (P-EGFR) and total EGFR levels in lysates of A549 cells infected with Kp52145 for 3 h (CON, uninfected control cells).
C. Immunoblot analysis of IκBα and tubulin levels in lysates of siRNA-transfected (AS-, AllStars control siRNA; EGFR siRNA, NOD1 siRNA, EGFR and NOD1 siRNAs) A549 cells which were left untreated, stimulated with IL-1β (1 ng ml⁻¹) for the indicated times, infected for 3 h, or infected for 3 h and then stimulated with IL-1β.
D. Immunoblot analysis of P-Akt, P-ERK, P-GSK3β levels and tubulin levels in lysates of A549 cells treated with EGFR inhibitor (AG1478, 5 μM, added 2 h before infection) or vehicle solution (DMSO) and infected with Kp52145 for the indicated times.
E. Immunoblot analysis of CYLD levels in lysates of A549 cells treated with AG1478 (5 μM, added 2 h before infection) or vehicle solution (DMSO) and infected with Kp52145 for 3 h. Membranes were reprobed for tubulin as a loading control.
In all panels data are representative of at least three independent experiments.
investigated the role of LPS O-polysaccharide, CPS and the major outer membrane protein OmpA on Kp52145-induced EGFR activation. The mutants lacking LPS O-polysaccharide or OmpA, strains 52O21 and 52OmpA2 respectively, induced the phosphorylation of EGFR to similar levels to Kp52145 (Fig. 9A). However, EGFR phosphorylation was not detected in cells infected with the CPS mutant, strain 52145-ΔwzaK2, hence suggesting that CPS could mediate the activation of EGFR (Fig. 9A). To sustain this notion, cells were exposed to increasing amounts of purified CPS isolated from Kp52145 and the phosphorylation of EGFR was analysed by immunoblot. CPS induced the phosphorylation of EGFR in a dose-dependent manner (Fig. 9B). We next examined whether purified CPS activates the PI3K–AKT–PAK4–ERK–GSK3β signalling pathway via EGFR. Western blot analysis demonstrated that indeed purified CPS induced the phosphorylation of AKT, ERK and GSK3β (Fig. 9C). Moreover, this occurred in an EGFR-dependent manner since pretreatment of cells with AG1478 abrogated CPS-triggered phosphorylation of the three kinases (Fig. 9C). CPS also increased the levels of CYLD although they were lower than those induced by Kp52145 (Fig. 9D). Altogether, these findings suggest that CPS could be the bacterial factor responsible for EGFR activation.

The classical pathway of activation of the EGFR is initiated through binding of ligands to its extracellular domain and the subsequent autophosphorylation of two EGFR molecules (Yarden and Sliwkowski, 2001). Ligands such as epidermal growth factor (EGF) and heparin binding EGF (HB-EGF) are synthesized as membrane-spanning molecules that are proteolytically cleaved by metalloproteases to become active (Yarden and Sliwkowski, 2001). Therefore the activation of EGFR could involve a metalloprotease-dependent activation involving the shedding of HB-EGF or autocrine/paracrine EGF-mediated activation of the receptor. To explore whether CPS may induce the release of EGFR ligands we asked whether bacteria or CPS cell-conditioned media trigger the phosphorylation of EGFR. However, incubation of cells with these conditioned media did not trigger the phosphorylation of the receptor (data not shown).

Given that we and others have shown that K. pneumoniae CPS can activate host cell signalling in a TLR4-dependent manner (Regueiro et al., 2008; Yang et al., 2011) and that TLR4 signalling has been associated with EGFR activation (Koff et al., 2008), we hypothesized that CPS may engage TLR4 to activate EGFR. To pursue this notion, we analysed the phosphorylation of EGFR in cells knockdown for TLR4 by siRNA. While CPS or Kp52145 induced the phosphorylation of EGFR in cells transfected with control siRNA, this was not the case in TLR4 knockdown cells (Fig. 9E). Likewise, CPS and Kp52145 failed to trigger the phosphorylation of EGFR in MyD88 knockdown cells (Fig. 9F). Altogether, these results suggest that a TLR4–MyD88-dependent signalling is involved in CPS-dependent phosphorylation of EGFR.

The facts that TLR4 signalling is coupled to the activation of the SRC kinase family (Gong et al., 2008) and that this family of kinases is involved in the phosphorylation of EGFR (Thuringer et al., 2011) led us to investigate whether SRC kinases mediate CPS-triggered phosphorylation of EGFR. Pretreatment of cells with the SRC inhibitor PP2 (Hanke et al., 1996) inhibited the CPS-dependent phosphorylation of EGFR (Fig. S5). Likewise, PP2 inhibitor also diminished Kp52145-induced EGFR phosphorylation (Fig. S5). Since the SRC family member c-SRC has been shown to participate in EGFR activation (Thuringer et al., 2011), we sought to determine whether c-SRC mediates K. pneumoniae-induced EGFR phosphorylation. Indeed, in cells knockdown for c-SRC neither CPS nor Kp52145 triggered the phosphorylation of EGFR (Fig. 9G). As expected, Western blot analysis revealed that Kp52145 and CPS induced the phosphorylation of c-SRC (Fig. 9H and I). However, this was not the case in cells knockdown for TLR4 (Fig. 9H and I).

Collectively, our findings revealed that Kp52145 CPS is the factor responsible for the activation of EGFR. Moreover, CPS-induced activation of EGFR is dependent on a TLR4–MyD88–c-SRC-dependent pathway. Nevertheless, purified CPS did not prevent IL-1β-induced nuclear translocation of the NF-κB p65 subunit (Fig. S6).

Among the described CPS K types of the serotyping scheme, types K1, K2, K4 and K5 are associated with severe infections in humans (Sahly and Podschun, 1997; Podschun and Ullmann, 1998; Sahly et al., 2008; Brisse et al., 2009). In fact, K. pneumoniae isolates from severe invasive infections express high levels of CPS and frequently belong to the CPS serotypes K1 or K2 (Sahly and Podschun, 1997; Podschun and Ullmann, 1998; Sahly et al., 2008; Brisse et al., 2009). Since Kp52145 expresses a CPS belonging to serotype K2, we asked whether K. pneumoniae strain expressing CPS of K1 serotype would elicit similar signalling pathway to CPS from Kp52145. CPS of K1 serotype was purified from strain NTUH-K2044, which is a reference strain of the highly virulent K1 isolates (Brisse et al., 2009). Western blot analysis revealed that NTUH-K2044 and its purified CPS induced the phosphorylation of EGFR (Fig. S7A). However, this was not the case in cells knockdown for TLR4 or c-SRC (Fig. S7B and S7C respectively). NTUH-K2044 and purified CPS also induced the phosphorylation of AKT, ERK and GSK3β in an EGFR-dependent manner since pretreatment of cells with AG1478 abrogated the phosphorylation of the three kinases (Fig. S7D). Purified CPS and NTUH-2044 also increased the levels of CYLD although the levels induced by the purified CPS were...
lower than those induced by NTUH-K2044 (Fig. S7E). Like the CPS of the K2 serotype, purified CPS of the K1 serotype did not prevent IL-1β-induced nuclear translocation of the p65 subunit in contrast to NTUH-2044 (Fig. S7F).

Discussion

We have recently shown that *K. pneumoniae* dampens the activation of inflammatory responses in host cells by targeting the activation of the NF-κB canonical pathway (Regueiro *et al.*, 2011). Our results revealed that *K. pneumoniae* takes control over cellular systems dedicated to control the host immune balance by engaging a NOD1-dependent pathway (Regueiro *et al.*, 2011) (Fig. 10). To identify additional factors involved in the anti-inflammatory effect exerted by *K. pneumoniae*, we standardized and applied an unbiased high-throughput siRNA gain-of-function screen interrogating the human kinome. Using this approach, we identified 17 kinases that when targeted by siRNA restored IL-1β-dependent NF-κB translocation in infected cells. Follow-up validation revealed that *K. pneumoniae* exploits an EGFR–PI3K–AKT–PAK4–ERK–GSK3β signalling pathway to attenuate the cytokine-dependent nuclear translocation of NF-κB (Fig. 10). This study represents the first comprehensive, functional genomics-driven identification of previously unknown host factors targeted by a human pathogen to downregulate the NF-κB signalling pathway.

Except for PAK4, none of the other high confident hits (ILK, CDK9, HGS, PKMYT1 and PRKACB) affected the activation of ERK during *K. pneumoniae* infection hence suggesting that additional pathways contribute to *K. pneumoniae*-induced anti-inflammation. Since this will be the subject of future studies, at present we can only speculate about their contribution to the anti-inflammatory phenotype. Only ILK, CDK9 and PRKACB have been implicated in NF-κB signalling in prior studies. There are almost no reports showing manipulation of these kinases by a bacterial pathogen. The cAMP-dependent protein kinase/protein kinase A (PKA) holoenzyme is composed of dimer catalytic subunits and dimer regulatory subunits, being PRKACB a catalytic subunit (Taylor *et al.*, 2008). PKA has been shown to affect NF-κB-dependent gene transcription by modulating the phosphorylation levels of the NF-κB subunit p65 at Ser-276 (Zhong *et al.*, 1997; 1998). However, the regulatory action seems to correlate inversely with the levels of the scaffold protein A kinase-interacting protein 1 (AKIP1) (Zhong *et al.*, 1997; Gao *et al.*, 2010; King *et al.*, 2011). It is then tempting to speculate that *K. pneumoniae* might affect the levels of AKIP1 to modulate the activity of PKA. We also validated two cyclin-dependent kinases (CDKs), CDK5 and CDK9. Despite the name, both are not directly involved in control of the cell cycle. Recently, both CDKs have been identified as modulators of TNFα-induced NF-κB-dependent gene expression (Choudhary *et al.*, 2011). Interestingly, CDK9 regulatory action is also related to the phosphorylation levels of the NF-κB subunit p65 at Ser-276 (Nowak *et al.*, 2008). In fact, it is known that NF-κB p65 subunit can be phosphorylated at multiple sites including Ser-276 (Viatour *et al.*, 2005). These phosphorylation events lead to an increase in nuclear translocation, DNA binding and/or transcriptional activity, through mechanisms not completely understood yet (Viatour *et al.*, 2005). Studies are ongoing to clarify the impact of *Klebsiella* infection on the phosphorylation levels of p65 and how this affects NF-κB activation. ILK is a β1 integrin cytoplasmic domain-interacting protein acting as a scaffold protein to connect integrins to the actin cytoskeleton and signalling pathways including NF-κB-dependent ones (Tan *et al.*, 2002; Hannigan *et al.*, 2005; Legate *et al.*, 2006; Holmes *et al.*, 2012). However, in contrast to our findings, the published studies support the notion that NF-κB activation is diminished in ILK knockdown cells (Tan *et al.*, 2002; Holmes *et al.*, 2012). In any case, our findings point out...
that *Klebsiella* may manipulate β1 integrin–ILK signalling cascade to dampen inflammatory responses. *Shigella flexneri* and *Yersinia enterocolitica* have been also reported to target integrin-dependent responses. Whereas *Shigella* hijacks integrins to prevent cell detachment (Kim *et al.*, 2009), *Yersinia*-triggered inflammatory responses depend on the engagement of integrins (Schulte *et al.*, 2000).
The PI3K–AKT and ERK-dependent signalling cascades play a central role in the regulation of a number of cellular functions including cell proliferation, metabolism, apoptosis and also inflammation. Moreover, deregulation of these cascades is associated with the development of several cancers. Therefore, it is not unexpected that pathogens target these proteins to subvert central cell functions. However, to the best of our knowledge, *K. pneumoniae* is the first pathogen targeting this pathway to block NF-κB activation. Moreover, our findings demonstrated a critical role of *K. pneumoniae*-induced inactivation of GSK3β for the anti-inflammatory effect. The fact that *K. pneumoniae* induces the phosphorylation of AKT and GSK3β in whole lungs (Hoogendijk et al., 2011) highlights the biological relevance of our *in vitro* findings. GSK3β is a point of convergence for numerous signalling pathways hence controlling multitude physiological process such as cell cycle, cell differentiation, cell adhesion and inflammation (Wang et al., 2011). In this context, GSK3β is emerging as a key hub integrating different signals to regulate inflammatory responses. In fact, inactivation of GSK3β augments anti-inflammatory responses while suppressing the production of pro-inflammatory mediators (Martin et al., 2005). Altogether, our findings support the notion that *K. pneumoniae* targets GSK3β to tip the host inflammatory response towards an anti-inflammatory state. In this regard, several reports have shown that a distinct feature of *K. pneumoniae* pneumonia is the high levels of the anti-inflammatory cytokine IL-10 and the low levels of inflammatory cytokines such as IL-12 and IL-6 (Yoshida et al., 2001; Lawlor et al., 2006). We are currently assessing whether *Klebsiella* inactivation of GSK3β is responsible for the cytokine milieu found in *Klebsiella*-infected animals.

Fig. 10. Working model depicting *K. pneumoniae* strategies to subvert the activation of NF-κB. This model is based on the findings reported in this manuscript and includes our previous observations (Regueiro et al., 2011). Kp52145 inhibits cytokine-dependent activation of the NF-κB canonical pathway in a process dependent on the activation of the deubiquitinase CYLD. In our previous study we showed that Kp52145 inhibits the activation of the deubiquitinase CYLD. In this work, we have shown that Kp52145 activates an EGFR–PI3K–AKT–PAK4–ERK–GSK3β signalling pathway to attenuate the cytokine-dependent nuclear translocation of NF-κB via CYLD. Kp52145-mediated EGFR activation is dependent on a TLR4–MyD88–c-SRC-dependent pathway. The model highlights that Kp52145-induced ERK phosphorylation is dependent on both EGFR and NOD1. Since our findings suggest that both receptors do not play redundant roles, the model proposes that the activation of both EGFR and NOD1 is required to reach the maximum levels of CYLD.
Perusal of the literature shows that the PI3K–AKT–GSK3β cascade also regulates these cellular functions. A tantalizing hypothesis could be that *K. pneumoniae* could also target these physiological processes in a PI3K–AKT–GSK3β–CYLD-dependent manner. Future studies will explore this hypothesis.

Further highlighting the important role of *Klebsiella* induction of CYLD expression on the host–pathogen interaction, in this work we have shown that *Klebsiella* activates the NF-κB canonical cascade in CYLD knockdown cells in a TLRs–MyD88-dependent manner. This is in agreement with the notion that TLR signalling controls the activation of host defence responses against *Klebsiella* (Happel *et al.*, 2003; Schurr *et al.*, 2005; Wieland *et al.*, 2010). Moreover, *in vitro* and *in vivo* approaches consistently sustain the idea that both TLR2 and TLR4 are implicated in sensing *Klebsiella* infections (this work and Regueiro *et al.*, 2006; 2008; Moranta *et al.*, 2010; Wieland *et al.*, 2010). Therefore, it is also likely that the engagement of a TLR–MyD88 pathway by Kp52145 underlies the degradation of IkBα observed in PK4 or ERK knockdown infected cells. It should be noted that pharmacologic inhibition of ERK activation prevents Kp52145-induced CYLD expression and that Kp52145-triggered ERK phosphorylation is impaired in PK4 knockdown cells. Our results also provide mechanistic information to our previous reports demonstrating *K. pneumoniae*-mediated inhibition of TLR signalling (Regueiro *et al.*, 2006; Moranta *et al.*, 2010). Contrary to our initial speculation that *Klebsiella* impairs TLR recognition (Regueiro *et al.*, 2006; Moranta *et al.*, 2010), the results shown in this work indicate that *Klebsiella* does engage TLRs although the pathogen blocks the TLR-dependent inflammatory signalling cascade in a CYLD-dependent manner.

Of particular interest is the identification of EGFR as a critical mediator in *K. pneumoniae*-induced activation of the PI3K–AKT–PK4–ERK–GSK3β signalling cascade. Only three bacterial pathogens, *Helicobacter pylori*, *Pseudomonas* spp. and *Haemophilus influenzae*, are known to activate EGFR (Zhang *et al.*, 2004; Keates *et al.*, 2007; Choi *et al.*, 2011; Xu *et al.*, 2011). In all cases, EGFR activation is linked to the launch of inflammatory responses (Zhang *et al.*, 2004; Keates *et al.*, 2007; Choi *et al.*, 2011; Xu *et al.*, 2011). In contrast, and to the best of our knowledge, *K. pneumoniae* is the first pathogen hijacking EGFR to block the activation of the NF-κB canonical pathway. Furthermore, this work is the first study showing a role for EGFR in the expression of CYLD. These results do not contradict our recent study showing that *K. pneumoniae* engages NOD1 to attenuate inflammatory responses (Regueiro *et al.*, 2011). In fact, our data revealed that in cells double knockdown for EGFR and NOD1 the anti-inflammatory effect is nearly abolished in contrast to what happens in the single knockdown cells hence suggesting that both receptors do not play redundant roles. At the very least our data suggest that the *K. pneumoniae*-induced ERK phosphorylation is dependent on both EGFR and NOD1-dependent signalling cascades (Fig. 10).

We were keen to identify the bacterial factor(s) responsible for EGFR activation. In this study, we demonstrate a role for CPS in the activation of EGFR via a TLR4–MyD88–c-SRC-dependent pathway. Of note, we and others have shown that CPS indeed elicits TLR4-dependent signals (Regueiro *et al.*, 2008; Yang *et al.*, 2011). We are aware that, despite extensive purification, our CPS preparations may still contain traces of proteins and LPS. In our efforts to rule out the effect of protein contaminants, the CPS preparation was repurified using a method established to remove proteins from polysaccharide preparations (Manthey *et al.*, 1994; Hirschfeld *et al.*, 2000). Although no proteins were detected in the repurified CPS preparation by SDS-PAGE and colloidal gold staining we cannot totally rule out that protein traces could be responsible, at least partially, for EGFR activation. The fact that strain 52O21, LPS O-polysaccharide mutant but expressing CPS, induced EGFR phosphorylation, whereas strain Kp52145-ΔwzaA2, CPS mutant but expressing wild-type LPS, did not induce such phosphorylation, strongly suggests that LPS traces are not responsible for CPS-induced EGFR activation. Since we showed in a previous study that CPS does not activate NOD1-dependent responses (Regueiro *et al.*, 2011), we propose that the reduced NF-κB activation characteristic of *K. pneumoniae* infections is the sum of, at least, two cascades: the CPS-dependent activation of EGFR signalling and the CPS-independent NOD1-signalling pathway (Fig. 10). Future studies will aim to identify *K. pneumoniae* factor(s) required to attenuate inflammatory responses in a NOD1-dependent manner. Nevertheless, and further confirming previous results (Regueiro *et al.*, 2011), purified CPS did not prevent IL-1β-triggered NF-κB activation. These findings are consistent with the concept that CPS is necessary but not sufficient to attenuate inflammation. The fact that CYLD levels were higher in infected cells than in CPS-challenged cells may indicate that a certain threshold of CYLD is required to effectively attenuate inflammation being the levels induced by the purified CPS not high enough. Biochemical studies are warranted to define the minimum CYLD levels necessary to prevent NF-κB activation.

Finally, it is worthwhile commenting on the clinical implication of our study. The frequent isolation of *K. pneumoniae* multidrug-resistant strains makes necessary to develop effective therapeutics based on new targets/approaches. EGFR as well as the PI3K–AKT–GSK3β cascade have been deeply studied due to their relevance
for many human cancers. As a result, several promising drugs have been developed or are being developed to antagonize these cascades. This offers the potential for pharmacological intervention by targeting the host side, a paradigm receiving attention in the field of infectious diseases (Schwegmann and Brombacher, 2008). Therefore, we propose that agents targeting these signalling pathways might provide selective alternatives for the management of \textit{K. pneumoniae} pneumonias.

**Experimental procedures**

**Bacterial strains and growth conditions**

\textit{Klebsiella} \textit{pneumoniae} strain 25745 is a clinical isolate (serotype O1:K2) previously described (Nassif et al., 1989; Cortes et al., 2002). Strains 52021, an isogenic LPS O-polysaccharide mutant (Cortes et al., 2002), 520mpA2, isogenic ompA mutant (Llobet et al., 2009), and 52145–Δwzzc, an isogenic mutant lacking capsule (Llobet et al., 2008), have been previously described. 52021 and 520mpA2 express similar amounts of CPS to the wild-type strain (Cortes et al., 2002; Llobet et al., 2009). NTUH-K2044 is a clinical isolate (serotype O1:K1) previously described (Fang et al., 2004; Hsieh et al., 2012). Bacteria were grown in Luria–Bertani medium at 37°C.

**Cell culture and infections**

\textit{A549} cells (ATCC CCL185; human lung carcinoma epithelial cell line) were grown as previously described (Regueiro et al., 2011). SIB01 cells were grown in RPMI 1640 medium supplemented with 0.3 g l\(^{-1}\) L-glutamine, 25 mM Hepes and 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified 5% CO\(_2\) incubator. Cells used for high-throughput screening and hit validation were always of the same passage, subcultured for 1 week after removal from liquid nitrogen storage.

Bacteria were prepared as described (Regueiro et al., 2011) and infections were performed using a multiplicity of infection of 150 bacteria per cell, unless otherwise indicated.

**Reagents**

Recombinant human IL-1β was purchased from Peprotech (UK) and was reconstituted in 0.1% IgG-free BSA (Sigma) and stored as single-use aliquots at −20°C. PP2, AKT X, AG1478, U0126 and LY294002 were from Calbiochem. Hoechst 33342 was from Invitrogen. Plasmids for transfection of mammalian cells were isolated from \textit{Escherichia coli} host DH5α and purified using ‘Endofree’ maxiprep kits (Qiagen). GSK3β-HA-WT and constitutively active GSK3β-HA-S9A constructs were obtained from Addgene (Catalogue Reference #14753 and #14754 respectively). Protein A/G sepharose was from Santa Cruz. Primary rabbit antibodies were from Cell Signaling (ixBα, P-PAK4; P-Erk, P-GSK3β, P-Akt), Millipore (EGFR, P-EGFR) or Santa Cruz (HA, p65). All primary antibodies were used at 1:1000 concentrations. Mouse monoclonal anti-tubulin antibody (1:3000) was from Sigma. HRP-conjugated goat anti-rabbit (1:20 000) and goat anti-mouse (1:1000) secondary antibodies used for immunoblots were from Fisher Scientific. Rhodamine Red-X and CY2-conjugated donkey anti-rabbit antibodies used for immunofluorescence were from Jackson ImmunoResearch.

**Small interfering RNA (siRNA)**

The siRNA library targeting 646 human kinases and kinase-related genes, distributed over 14 master plates in 96-well microtitre plate format, was from Qiagen (Human Kinase siRNA Set V1.0). siRNAs used for hit validation (three ‘Flexi’-master plates), AllStars negative control (AS), siRNAs targeting CDK9, HGS, ILK, PAK4, PKMYT1, PKRACB, MyD88, ERK1/2, EGFR (3’ UTR EGFR) and a toxic siRNA control targeting PLK1 were also purchased from Qiagen. Stealth siRNA targeting NOD1 and TLR4 was from Invitrogen. Further details and sequences can be found in Table S2.

**siRNA knockdown efficiency**

Knockdown efficiency of the siRNAs used in the in-depth analysis was determined on protein level by immunoblot analysis (Fig. S8A; ERK1/2, MyD88, PAK4, CYLD, EGFR, c-SRC) and/or on transcript level by real-time quantitative PCR (RT-qPCR) (Fig. S8B; PAK4, ILK, CDK9, HGS, PKRACB, PKMYT1; Fig. S8C; ERK1, ERK2) as described previously (Moranta et al., 2010; Regueiro et al., 2011). Sequences of the oligonucleotide primers used for qPCR can be found in Table S2. The knockdown efficiency of the siRNAs targeting NOD1, TLR2 and TLR4 has been verified previously (Moranta et al., 2010; March et al., 2011; Regueiro et al., 2011).

**High-throughput siRNA screening**

(i) \textit{Library transfection.} Two picomoles of dried down siRNAs, spotted in each of the inner 60 wells of each plate (48 library +12 control siRNAs; see Fig. 1C), were resuspended in 10 μl of Opti-MEM, mixed with 1 μl of HiPerFect lipofection reagent (Qiagen) diluted in 25 μl of Opti-MEM and incubated at RT for 10 min, after which 2500 A549-SIB01 cells in 75 μl of RPMI with 14% FCS were added and mixed (final volume 100 μl; siRNA 20 nM). After 10 min incubation the plates were transferred to a humidified 37°C CO\(_2\) incubator for 7 h until the p65 translocation assay was carried out.

(ii) \textit{Infection.} A549-SIB01 cells after 72 h transfection (~25 000 cells per well) were washed with PBS and then 100 μl of medium (RPMI 1640 with 10% FCS) for non-infected wells or 100 μl of medium containing bacteria (adjusted to a m.o.i. of ~200:1) was added. After incubation for 3.5 h at 37°C in a humidified CO\(_2\) incubator, IL-1β (final concentration 10 ng ml\(^{-1}\) in 10 μl of medium) was added to the cells. After 30 min incubation at 37°C the supernatants were aspirated and the cells fixed with 4% paraformaldehyde in PBS supplemented with 4 μg ml\(^{-1}\) Hoechst 33342. After 30 min incubation at RT the plates were washed twice with PBS and then 100 μl of a 14 mM ammonium chloride in PBS solution was added. This was either directly followed by image acquisition, or plates were stored at 4°C for later processing.

(iii) \textit{Image acquisition and analysis.} Images in two non-overlapping fields were recorded using an automated microscope.
(Olympus) with a 4× magnifying objective with GFP and DAPI filter sets. For the hit validation, images from four fields per well were recorded using a 10× objective. Image analysis was carried out using the scan-R software package (Olympus) with a customized algorithm essentially as previously described (Bartfeld et al., 2010).

(iv) Statistical analysis. The assay quality Z-factor was calculated from % translocation data of the positive controls (uninfected wells stimulated with IL-1β) and the data of the negative controls (infected wells stimulated with IL-1β). The data of percentage of translocating cells (% translocation) was normalized using the Z-score method relative to the mean of the 48 library wells of a plate. For a given well/target the Z-score was calculated by subtracting the standard deviation value for the library wells on that plate from the % translocation value of the well and dividing by the standard deviation value for the library wells.

Transfection conditions

Unless stated otherwise, transfection of A549 cells with siRNAs or plasmids was carried out using Lipofectamine 2000 (Invitrogen) lipofection reagent according to manufacturer’s instructions. For transfection of siRNAs, 4× 10⁴ (24-well format), 8× 10⁴ (12-well format) or 1.6× 10⁵ (six-well format/35 mm dish) cells were transfected in suspension with 20 nM siRNA using 1.2 µl (24-well), 2 µl (12-well) or 4 µl of Lipofectamine 2000 (six-well) in a final volume of 0.5 ml, 1 ml or 2 ml respectively. Experiments were carried out 48 h after transfection. For transfection of plasmids, 1× 10⁶ (12-well) or 2× 10⁶ (six-well/35 mm dish) cells were seeded and the next day transfected with 3 µl (12-well) or 6 µl of Lipofectamine 2000 (six-well) and 2 (12-well) or 4 µg (six-well) of plasmid in a final volume of 1 or 2 ml respectively. Experiments using plasmid-transfected cells were carried out the 24 h after transfection.

Immunofluorescence microscopy

Immunofluorescence microscopy of SIB01 (without additional anti-p65 staining) or transfected A549 cells grown on 13 mm circular coverslips in 24-well plates was carried out as described (Regueiro et al., 2011). p65 NF-xB subunit was stained with anti-p65 antibody (1:200; Santa Cruz Biotechnology). Cy2 or Rhodamine Red-X-conjugated donkey anti-rabbit antibodies (Jackson Immunological) were diluted 1:200. Immunofluorescence was analysed with a Leica CTR6000 fluorescence microscope.

Immunoblot analysis

Proteins were resolved by standard 6%, 8% or 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked with 4% skim milk in PBS or TBST and protein bands were detected with specific antibodies using SuperSignal (PIERCE) or Luminata Forte (Millipore) chemiluminescence reagents and a GeneGnome chemiluminescence imager (Syngene). Tubulin levels were routinely determined on the same membranes to ensure comparable loading. To detect multiple proteins, membranes were reprobed after stripping of previously used antibodies using RESTORE (PIERCE) or a pH 2.2 glycine-HCl/SDS buffer.

Immunoprecipitation of EGFR

Phosphorylated and total EGFR was detected in lysates of A549 cells grown in six-well plates following immunoprecipitation of total EGFR using a modification of a protocol described by Choi et al. (2011). In brief: cells were lysed in IP lysis buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1 mM EDTA, 400 mM Na3VO4, 2.5 mM phenylmethylsulfonyl fluoride) containing 0.1% Triton X-100 and incubated on ice for 40 min. Four micrograms of rabbit anti-EGFR antibody were added to the cell lysate supernatant, and incubated by rotation overnight at 4°C. Each sample was mixed with protein A/G-Sepharose and rotated at 4°C for 3 h. The mixture was washed three times with IP lysis buffer after which 40 µl of 2× SDS sample buffer was added. Immunoprecipitates were collected by centrifugation and subjected to 8% SDS-PAGE and semi-dry electrotransferred onto a PVDF membrane using as transfer buffer SDS-PAGE urea lysis buffer [a freshly prepared 1:1 mix of 1× SDS running buffer (12 mM Tris, 96 mM glycine, 0.1% SDS) and urea lysis buffer (10 mM Na2HPO4, 1% β-mercaptoethanol, 1% SDS, 6 M urea)] (Abeyrathne and Lam, 2007).

CPS purification

CPSs from Kp52145 and NTUH-K2044 were purified by the phenol-water method as previously described (Llobet et al., 2008; Regueiro et al., 2008). To purify CPS preparations, they were dispersed (final concentration 10 mg ml⁻¹) in 0.8% NaCl–0.05% NaN3–0.1 M Tris-HCl (pH 7) and digested with nucleases [50 mg ml⁻¹ of DNase II type V and RNase A (Sigma Chemical, St. Louis, MO, USA)] for 18 h at 37°C. Proteinase K was added (50 mg ml⁻¹; E. Merck, Darmstadt, Germany), and the mixtures were incubated for 1 h at 55°C and for 24 h at room temperature. The proteinase K digestion was repeated twice and the polysaccharides were precipitated by adding five volumes of methanol plus 1% (v/v) of a saturated solution of sodium acetate in methanol. After incubation for 24 h at –20°C, polysaccharides were recovered by centrifugation, and dissolved in distilled water. The LPS was removed by ultracentrifugation (105 000 g, 16 h, 4°C) and samples were freeze-dried. The enzymatic treatment and ultracentrifugation steps were repeated another time. CPSs were further repurified by the method described by Hirschfeld et al. (2000). This method is widely used to remove proteins from polysaccharide preparations. Analysis of the repurified CPSs by SDS-PAGE followed by silver staining revealed no traces of contaminants proteins (data not shown). SDS-PAGE-resolved preparations were transferred to PVDF membrane which was stained with colloidal gold to visualize proteins (Manthey et al., 1994). As before no proteins were detected in the repurified CPS preparations. LPS presence was determined by measuring the 3-deoxy-d-manno-2-octulosonic acid (Kdo) content by the thio-barbituric acid method modified to correct interference due to deoxy sugerins (Díaz-Aparicio et al., 1993). Kdo content was less than 0.1%.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** NF-κB readout using A549 p65–GFP reporter cells automated image analysis. A. For screening purposes a modified A549 cell line with a p65–GFP reporter construct (SIB01 cell line) was used to monitor the translocation of GFP into the nucleus upon activation with IL-1β. The ratio of GFP signal in the nucleus versus the cytoplasm was taken as the level of cell activation. B. To quantify cell populations, first cells were fixed and stained with Hoechst 33342 and images acquired. Second the images were subjected to automated image analysis. Based on the Hoechst signal, cell nuclei were detected as main object ‘nucleus’. Based on the features area and signal intensity only for main objects that met nuclei-like criteria the subobjects ‘core’ and ‘nucleus’ were applied. Based on the ratio of the measured GFP level and down-regulation of cytokine production by *Klebsiella pneumoniae*. **Fig. S2.** Knockdown of some validated targets does not affect *K. pneumoniae*-induced ERK phosphorylation. Immunoblot analysis of P-ERK and tubulin levels in lysates of siRNA-transfected A549 cell infected with KP52145 for the indicated times. siRNA targets are indicated (top row of each panel; AS-,
AllStars control siRNA). Data are representative of at least three independent experiments.

**Fig. S3.** Effect of AKT inhibitor on *Klebsiella pneumoniae*-induced phosphorylations of ERK and GSK3β. Immunoblot analysis of P-ERK, P-GSK3β and tubulin levels in lysates of AKT inhibitor (AKT, 30 μM, added 1 h before infection) or vehicle solution (DMSO)-treated A549 cells infected with Kp52145 for the indicated times. In all panels data are representative of at least three independent experiments.

**Fig. S4.** Effect of TLR2 and TLR4 on *Klebsiella pneumoniae*-induced phosphorylation of EGFR. Immunoblot analysis of P-EGFR and total EGFR levels in lysates of siRNA-transfected (AS, AllStars control siRNA; TLR2 siRNA, TLR4 siRNA, CYLD siRNA, CYLD and TLR2 siRNAs; and CYLD and TLR4 siRNAs) A549 cells which were infected with Kp52145 for 3 h or left uninfected. Membranes were reprobed for tubulin as a loading control. In all panels data are representative of at least three independent experiments.

**Fig. S5.** Effect of SRC inhibitor on *Klebsiella pneumoniae*-induced phosphorylation of EGFR. Immunoblot analysis of P-EGFR and total EGFR levels in lysates of SRC inhibitor (PP2, 40 μM, added 1 h before infection) or vehicle solution (DMSO)-treated A549 cells infected with Kp52145 (Kp), challenged with purified CPS (100 μg ml⁻¹) for 3 h (CPS) or left untreated (mock). Data are representative of three independent experiments.

**Fig. S6.** Effect of CPS purified from *K. pneumoniae* 52145 on NF-κB activation. Immunofluorescence microscopy of A549 cells grown on glass coverslips. Cells were treated with CPS (100 μg ml⁻¹) for 3 h (+ CPS) or left untreated (– CPS) (mock), and then stimulated with IL-1β (10 ng ml⁻¹) for 20 min. Coverslips were stained with anti-p65 NF-κB subunit and rhodamine Red-X-conjugated donkey anti-rabbit antibody. Scale bar, 20 μm.

**Fig. S7.** Purified CPS from *K. pneumoniae* NTUH-K2044 activates the EGFR–PI3K–AKT–ERK–GSK3β signalling cascade.

A. Immunoblot analysis of P-EGFR and total EGFR levels in lysates of A549 cells infected with NTHU-K2044 (NTUH; wild-type strain) or treated for 3 h with 100 μg ml⁻¹ CPS purified from NTHU-K2044.

B. Immunoblot analysis of P-EGFR and total EGFR levels in lysates of siRNA-transfected (AS, AllStars control siRNA; TLR4 siRNA) A549 cells infected with NTHU-K2044 (NTUH) for 3 h or challenged with purified CPS (100 μg ml⁻¹) for 3 h.

C. Immunoblot analysis of P-EGFR and total EGFR levels in lysates of siRNA-transfected (AS, AllStars control siRNA; c-SRC siRNA) A549 cells infected with NTHU-K2044 (NTUH) for 3 h or challenged with purified CPS (100 μg ml⁻¹) for 3 h.

D. Immunoblot analysis of P-Akt, P-ERK, P-GSK3β levels and tubulin levels in lysates of A549 cells treated with EGFR inhibitor (AG1478, 5 μM, added 2 h before infection) or vehicle solution (mock; DMSO); and infected with NTHU-K2044 (NTUH) for 3 h or challenged with purified CPS (100 μg ml⁻¹) for 3 h.

E. Immunoblot analysis of CYLD and tubulin levels in lysates of A549 cells infected with NTHU-K2044 (NTUH) for 3 h or challenged with purified CPS (100 μg ml⁻¹) for 3 h.

F. Immunofluorescence microscopy of A549 cells grown on glass coverslips. Cells were treated with CPS (100 μg ml⁻¹) for 3 h (CPS); infected with NTHU-K2044 (NTUH) or left untreated (mock), and then stimulated with IL-1β (10 ng ml⁻¹) for 20 min. Coverslips were stained with anti-p65 NF-κB subunit and rhodamine Red-X-conjugated donkey anti-rabbit antibody. Scale bar, 20 μm.

**Fig. S8.** siRNA knockdown efficiency.

A. Immunoblot analysis of EGFR, MyD88, ERK, CYLD, PAK4, c-SRC levels in lysates of siRNA-transfected (AS, AllStars control siRNA; EGFR siRNA, MyD88 siRNA, ERK siRNA, CYLD siRNA, PAK4 and c-SRC siRNA) A549 cells. Membranes were reprobed for tubulin as a loading control.

B. Transcript levels of indicated targets relative to AllStars control siRNA-transfected cells, as determined by RT-qPCR and normalization to GAPDH transcript levels.

C. Levels of ERK1 and ERK2 transcripts in ERK knockdown cells as determined by RT-qPCR and normalization to GAPDH transcript levels. Data are representative of three independent experiments in all panels.

**Table S1.** Targets selected for hit validation.

**Table S2.** Description of reagents (siRNA, primers and antibodies) used in this study.