Metalloprotease Type III effectors that specifically cleave JNK and NF-κB

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Table S1 – The strains used in this study

Name	Description	Reference or source
E2348/69	EPEC wild-type	J. Kaper
SN1961	E2348/69 Δ*escV*::*kan*	(Nadler et al., 2006)
EM3321	E2348/69 ΔPP2::*kan*	(Nadler et al., 2010)
EM3331	E2348/69 ΔPP6::*kan*	(Nadler et al., 2010)
EM3345	E2348/69 ΔIE5::*kan*	(Nadler et al., 2010)
EM3347	E2348/69 ΔIE6::*kan*	(Nadler et al., 2010)
EM3325	E2348/69 ΔPP4::*kan*	(Nadler et al., 2010)
SK4240	E2348/69 ΔIE6::*cm* ΔPP4::*kan*	This study
KB4298	E2348/69 Δ*nleD*::*kan*	This study
KB4296	E2348/69 Δ*nleC*::*kan*	This study
ICC168	*Citrobacter rodentium* (CR) wild-type	(Wiles et al., 2004)
BL21(DE3)	F-, ompT, hsdSβ(rβ-mβ-), dcm, gal, (DE3) tonA	Novagen
EM3326	E2348/69 ΔIE2::*cm*	(Nadler et al., 2010)
KB4309	E2348/69 ΔIE2::*cm* Δ*nleBE*$_{IE6}$::*tet*	(Nadler et al., 2010)
KB4325	E2348/69 ΔIE2::*cm* Δ*nleBE*$_{IE6}$::*tet* Δ*nleC*::*kan*	This study
KB4329	E2348/69 ΔIE2::*cm* Δ*nleBE*$_{IE6}$::*tet* Δ*nleCD*::*kan*	This study

Table S2 – The plasmids used in this study

Name	Description	Reference or source
pSA10	pKK177-3 derivative containing *lacI*^{q}	(Schlosser-Silverman et al., 2000)
pKB4345	pSA10 encoding *nleD* from EPEC	This study
pLG4457	pSA10 encoding *nleD*-E143A from EPEC	This study
pKB4505	pSA10 encoding *nleD1* from CR	This study
pKB4506	pSA10 encoding *nleD2* from CR	This study
pSC4141	A plasmid expressing *mCherry* deleted of its stop codon and with *ScaI* site added	(Nadler et al., 2010)
pLG4419	pSC4141 encoding *nleD* from EPEC	This study
pLG4477	pSC4141 encoding *nleD*-E143A from EPEC	This study
pCX341	Vector for formation of fusions with the *blaM* reporter	(Mills et al., 2008)
pME3654	pCX341 encoding *nleD-blaM*	This study
pKB4344	pSA10 encoding *nleC*	This study
pKB4403	pSA10 encoding *nleC*–E184A	This study
pSC4104	pSA10 containing *nleBE* IE6-6His with its promoter region	(Nadler et al., 2010)

pHG3656	pCX341 encoding *nleG-blaM*	This study
pET28a	Expression vector	Novagen
pRL-TK	Constitutively expressing renilla luciferase vector	Promega
pNFkB-luc	A plasmid expressing *luc* under NF-κB-dependent promoter	(Yaron et al., 1998)
pKB4745	pEGFP-N1 (clontech) with nleD	This study
pKB4746	pET14b (Novagen) with p65 1-210	This study
pHA-JNK	Vector for expression of HA-tagged JNK	(Kallunki et al., 1994)
pME3660	pCX341 encoding *nleC-blaM*	This study
pKB4407	pCX341 encoding *nleC*–E184A *-blaM*	This study
pLG4484	pCX341 encoding *nleD*–E143A *-blaM*	This study

Table S3 – The primers used in this study

Name	Sequence	Usage
Primers used for cloning		
*nleD*_into_pSA10_F (1075)	AGAATTCATGCGCCCTACGTCCCTCAAC	pKB4345, pKB4745 (EcoRI)
*nleD*_into_pSA10_R (1076)	ACCGTCGACCAGCTAAAGCAATGGATGCAG	pKB4345 (SalI)
nleD1 Citro into pSA10 F (1173)	AGAATTCCTTAATGCGCCCTACATCCC	pKB4505 (EcoRI)
nleD1 Citro into pSA10 R (1174)	ACCGTCGACCAACGTAGCTAAAGTAACGG	pKB4505 and pKB4506 (SalI)
nleD2 Citro into pSA10 R (1175)	AGAATTCATGGTATTTTCAGCCAAACC	pKB4506 (EcoRI)
nleD into pSC4141 F (1105)	ATCCTGAGTACTCGATGCGCCCTACGTCCCTC	pLG4419and pLG4477 (ScaI)
nleD into pSC4141 R (1106)	GCATGCGGCCGCCTAAAGCAATGGATGCAGTC	pLG4419 and pLG4477 (NotI)
nleD Fw (831)	GGTTGGGTACCATGCGCCCTACGTCCCTCAA	pHG3654 (KpnI)
nleD Rv (832)	GCGAATTCCCAAGCAATGGATGCAGTCTTAC	pHG3654 (EcoRI)
nleC_into_pSA10_F (1073)	AGAATTCCATGAAAATTCCCTCATTACAG	pKB4344 (EcoRI)
nleC_into_pSA10_R (1074)	ACCGTCGACTCATCGCTGATTGTGTTTGTC	pKB4344 (ScaI)
nleGFw (840)	GGTTGGGTACCATGCCATCATTAGTTTCAGGTA	pHG3656 (KpnI)
nleGRv (841)	GCGAATTCCCCTTATCCTTTATGACAAAGTTTC	pHG3656 (EcoRI)
p65 1-210 F (1235)	AATGCACATATGGACGAACTGTTCCCCC	pKB4746 (NdeI)
p65 1-210 R (1236)	ACTCGAGTTAATCCCCACCGAGGCAGC	pKB4746 (XhoI)
nleD into EGFP N1 R (1226)	CGGTCGACAGCAATGGATGCAGTCTTAC	pKB4745 (SalI)
Primers for deletions		
nleB_tet_F (946)	CCGTCTAAATGACGGGGCGATATTAACATGATTAGAACAAGAGGAATTTTCAAGAGGGTCATTATATTTCG	KB4309
nleE_tet_R (949)	TAATGAAATAGAATTAATTTTAGCCCCCCTACACAAGTGGCTGAGCTTCTACTCGACATCTTGGTTACCG	KB4309
nleC KO F (1060)	TTGTTGTAATAAATGATTTGCAGGGTATTAGATATAAACATGAAAATTCCCGTGTAGGCTGGAGCTGCTTC	KB4325
nleC KO R (1061)	TAAAATGTATGAATAGTAACCTTATGTCACTGCA	KB4325

	AAGACGAATCATCGCTGCATATGAATATCCTCCTTAG	
8M NO (K/O) F (628)	TTAATTATCAGTAAATTAATGTAAGTAGGTCATTATTAGTCAAAATAAAAGTGTAGGCTGGAGCTGCTTC	SK4240
nleD KO F (1062)	TAAACAGGCTGCTAATAAGTAGCATTCTCAGGAGTCCTGATGCGCCCTACGGTGTAGGCTGGAGCTGCTTC	KB4298
8M NO (K/O) R (629)	TGTGTGCCTCCGTTTGCCCCACCAATATATATTCAGCACAAGAAACACAGCATATGAATATCCTCCTTAG	SK4240, KB4298
Primers used for Mutagenesis		
nleD mut1 F (1151)	GACTATTGTTTTTCATGCGTTGCTCCATGTTTTCC	*nleD* E143A
nleD mut1 R (1152)	GGAAAACATGGAGCAACGCATGAAAAACAATAGTC	*nleD* E143A
nleC mut2 F (1095)	CAGGAAGGACTGATTCACGCGATTATTCATCATGTTAC	*nleC* E184A
nleC mut2 R (1096)	GTAACATGATGAATAATCGCGTGAATCAGTCCTTCCTG	*nleC* E184A

List of antibodies used in this study and working conditions:

1. Anti-p65-C-terminus domain (SC-372, Santa Cruz, 1:1000 in TBS)
2. Anti-p65-N-terminus domain (SC-109, Santa Cruz, 1:1000 in TBS)
3. Anti-JNK (554285, BD Pharmingen, 1:1000 in TBS)
4. Anti-HA,
5. Anti-6His (27471001, GE Healthcare, 1:3000 in TBS)
6. Anti-ERK (m5670, Sigma, 1:2500 in TBS)
7. Anti-c-Jun (H79, SCBT)
8. Anti-phospho-c-Jun (Cell Signaling)

Protein expression and purification

Plasmids expressing His-tagged NleC, NleC-E184A, NleD and $p65_{1-210}$ were transformed into *E.Coli* BL21 (DE3) and bacteria were grown to mid-exponential phase at which their expression was induced by incubation with either 1 mM IPTG at

20° C for 18 hours (NleC and NleC-E182A) or by the auto induction protocol at the same physical settings (Studier, 2005). Cells were collected by centrifugation at 6000 rpm for 7 min at 4° C after which the pellet was resuspended with binding buffer (20 mM Imidazole, 300 mM NaCl, 20 mM TRIS, pH 8, 0.02% Triton X-100). 10 mM, 6 mM and 3mM β-mercaptoethanol were added to the binding buffer of $p65_{1-210}$, NleC and NleC-E184A, respectively. Suspensions were lysed using French Press (Thermo Scientific) and centrifuged at 45,000 rpm for 45 min at 4° C. Protein batch binding was conducted by applying the supernatant to a Ni-NTA beads (Novagen), followed by 3 washing steps on a dripping column: step 1 buffer (25 ml of 300 mM NaCl, 20 mM Imidazole, 20 mM TRIS, pH 8), step 2 buffer (50 ml of 600 mM NaCl, 30 mM Imidazole, 20 mM TRIS, pH 8) and step 3 buffer (25 ml of 300 mM NaCl, 40 mM Imidazole, 20 mM TRIS, pH 8). Elution was performed in the presence of elution buffer (50 mM NaCl, 300 mM Imidazole, 20 mM TRIS, pH 8) after which eluted samples were equilibrated overnight against dialysis buffer (50 mM NaCl, 20 mM TRIS, pH 8) at 4° C with 5000 MW cutoff SnakeSkin® (Thermo Scientific). Equilibrated samples were purified with Mono-Q anion exchange column (GE healthcare) utilizing linear gradient with elution buffer (2M NaCl, 20 mM TRIS, pH 8). Selected fractions were further purified by size exclusion column (GE healthcare) equilibrated against 50 mM NaCl, 20 mM TRIS, pH 8. The collected fractions were pooled and concentrated to 44 $mg \cdot ml^{-1}$, 34.5 $mg \cdot ml^{-1}$, 16 $mg \cdot ml^{-1}$ and 8 $mg \cdot ml^{-1}$ (NleC, NleC-Mut, NleD and $p65_{1-210}$, respectively).

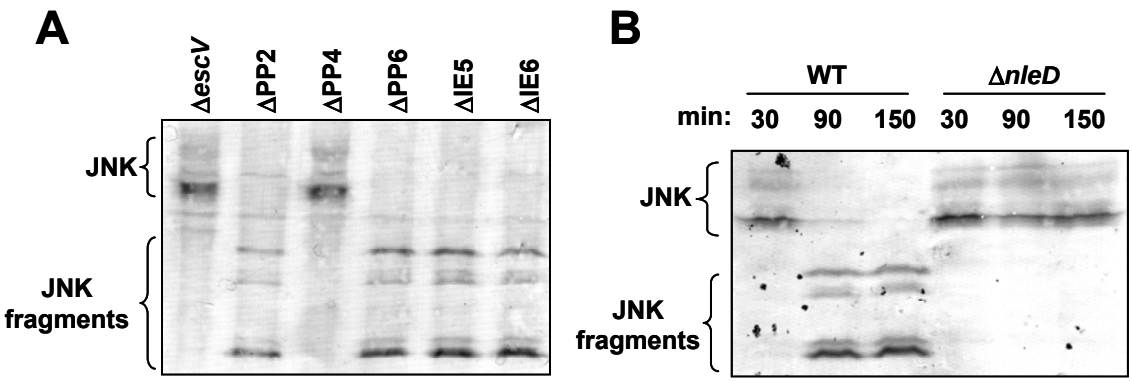

Fig S1. JNK degradation upon infection with different EPEC mutants

A. EPEC deleted of the PP4 chromosomal region is deficient in inducing JNK degradation. HeLa cells were infected for 3 h with different EPEC strains containing deletion of large chromosomal regions (the deleted regions are indicated above the lanes). Proteins were then extracted and subjected to Western blot analysis with anti-JNK antibody. The locations of the intact and fragmented JNK proteins are indicated. Mutant with inactivated TTSS (Δ*escV*) was used as negative control.

B. Complete JNK degradation by EPEC. HeLa cells were infected with EPEC for different periods with wild type EPEC (WT) or *nleD* mutant (Δ*nleD*), as indicated above the lanes. Proteins were then extracted and subjected to Western blot analysis with anti-JNK antibody. The locations of the intact and fragmented JNK proteins are indicated.

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EPEC        MRPTSLNLVLHQS---STSSSMSDTDIESLVKASSVQWIKNNPQLRFQGTDHNIYQQIEA 57
EHEC        MRPTSLNLVLHQS---SRSSSMSDTDIESLVKASSVQWIKNNPQLRFQGTDHNIYQQIEA 57
CR          MRPTSLNLTLPSLPLPSSSNSISATDIQSLVKMSGVRWVKNNQQLCFHGTDLKIYQHLEA 60
SE          ---------------------------------MRWIKNNQQLSFHGTDHKIYQQLEA 25
HD          MR-RALKLNLTPLS--FSSSSNSDTDIQSLVKKSSVNWIKNNHQLSFDGTNKNIYQRLEK 57
                                              :.*:*** ** *.**: :***::*

EPEC        ALDKIGSTETGRVLLNAIESISRLKSETVVIHLNSSRLGVMAHRDIDAENHRGTGSDFHC 117
EHEC        ALDKIGSTETGRVLLNAIESISRLKSETVVIHLNSSRLGVMAHRDIDAENHRGTGSDFHC 117
CR          ALDKIESTDTGRTLLNCIELTSRLKSEKLAIHLDSAELGVIAHCNADAENSRGTGSDFHC 120
SE          ALDKIESTDTGRILLKCIELTSQLKSEKLAIHLNCAELGVVAHCNTDAENARGTGSDFHC 85
HD          ALDKIESTETGKELLNCIESVSRLKSEKLIIRLDSTELGVTAHCAENAENFRGTGSYLHC 117
            ***** **:**: **:.**  *:****.: *:*:.:.*** **   :*** ***** :**

EPEC        NLNAVEYP-CGEGISVVDFHATIVFHELLHVFHNLNGERLKVESSRPESQKYSPLLLEEA 176
EHEC        NLNAVEYP-CGEGISVVDFHATIVFHELLHVFHNLNGERLKVESSRAESQKYSPLLLEEA 176
CR          NLNAVEYP-CGQGISLVDFHACIVFHELLHVFHNLNGERLKVESSQPELQTHSPLLLEEA 179
SE          NLNAVAYP-CGQGISLVDFHACIVFHELLHVLHNLNGERLKVESSQPESQTHYPFLLEEA 144
HD          NLNSVQEESIGKGIEQTELDACIVFHELLHVFHNLNGERLKVAVFQPEVETYSPFLLEEA 177
            ***:*     *:**. .::.* *********:**********   :.* :.: *:*****

EPEC        RTVGLGAFSEEVLSENKFREEIGMPRRTSYPHDSALIHDDNTVSLGFQQVRLHPLL 232
EHEC        RTVGLGAFSEEVLSENKFHEEIGMPRRTSYPXDSALIHDDNTVSLGFQQVRLHPLL 232
CR          RTVGLGAFSEEVLSENKFREEIGMPRRTFYPHDSSLIHDDNTVTQGFQRKKLHPLL 235
SE          RTVGLGSFSEEVLSENKFREEIGVPRRTFYPRDPYLIHDDNTVTQGLQRKKLHPLL 200
HD          RTVGLGSFSEEVFSENKFREEIGIPRRVSYAHESSLIHDDNTFTMFFENKQSHPLL 233
            ******:*****:*****:****:***. *. :. *******.:  ::. : ****
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Fig. S2. Multiple alignment of NleD homologs

The NleD protein sequences of EPEC (*Escherichia coli* O127:H6 str. E2348/69), EHEC (*Escherichia coli* O157:H7 EDL933), CR (*Citrobacter rodentium* ICC168 – has two copies of the *nleD* gene, encoding for an identical protein), SE (*Salmonella enterica* subsp. arizonae serovar 62:z4,z23:--) and HD (Candidatus *Hamiltonella defensa* 5AT) were aligned using ClustalW2. The HExxH Zn-metalloprotease motif is highlighted.

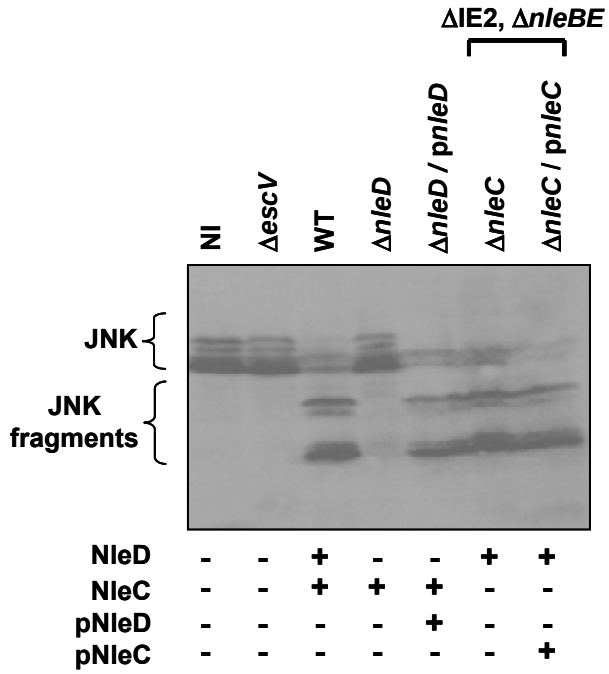

Fig. S3. NleD is required for EPEC-induced cleavage of JNK, *in vivo*, upon infection of Caco2 cells

Caco2 cells were infected for 3 h with different EPEC strains as indicated above the lanes or remain non infected (NI). Proteins were then extracted and subjected to Western blot analysis with anti-JNK antibody. The locations of the intact and fragmented JNK proteins are indicated. Mutant with inactivated TTSS (Δ*escV*) was used as negative control. In addition *nleC* mutant and a complimented mutant were used to show that NleC has no affect on JNK. The expression of native or plasmid expressed NleC and NleD is indicated below the blot. Expression is indicated by (+) and lack of expression by (-).

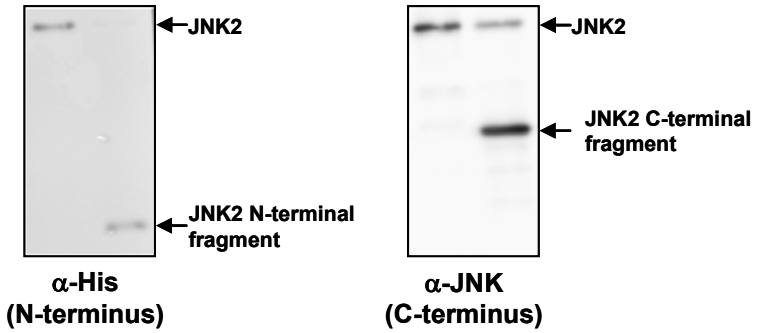

Fig. S4. Co-precipitation of the N- and C- terminal fragments of cleaved 6His-JNK2

Purified 6His-JNK2 was treated with Purified NleD to allow cleavage and the reaction products were further purified by gel filtration. Next, the proteins in a fraction containing the cleaved JNK2 were pulled down by Agaros-Ni beads, washed and eluted. The eluted proteins were analyzed by SDS-PAGE and Western blot using anti-6His (recognizing the N-terminus fragment of 6His-JNK2) and anti-JNK (recognizing the C-terminus fragment of JNK2). The locations of the JNK fragments and intact JNK2 are indicated. The remains of un-cleaved JNK2, which were seen with the anti-JNK, were not detected with the anti-6His antibody since the latter is less sensitive.

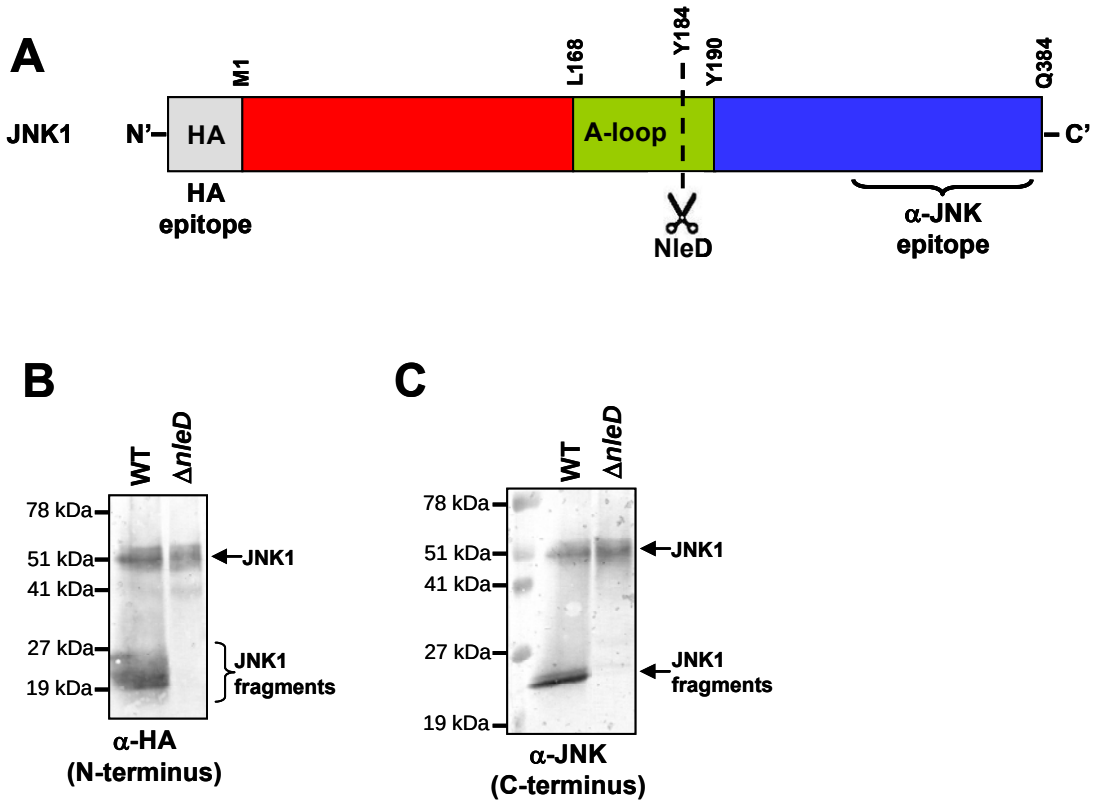

Fig. S5. NleD induces cleavage of HA-JNK1, *in vivo,* at its activation loop

HEK293 cells were transfected with a plasmids expressing JNK1, N-terminally tagged with the HA epitope. After 25 h, the cells were infected with wild type EPEC, or with EPEC *nleD* mutant and after 2.5 h infection the proteins were extracted from the infected cells and HA-JNK was immunoprecipitated with anti-HA antibodies. The precipitated proteins were analyzed using Western blot with either anti-HA; recognizing the N-terminal of HA-JNK1(B), or anti-JNK; recognizing the C-terminal of HA-JNK1 (C). Analysis with the anti-HA and anti-JNK antibodis identified N-terminal fragment of ~23 kD in size, and C-terminal fragments of ~24 kD in size. The latter is shorter from the JNK2 C-terminus fragment seen in Fig. 2, since JNK1 C-terminus region is shorter then that of JNK2. These results indicate that the C-terminal portion of the clipped HA-JNK1 remained in complex, and co-precipitated, with the HA-tagged N-terminus fragment. Based on the predicted size of the C- and N- terminal fragments generated by NleD digestion we estimated that, like in the case of JNK2 (Fig 2), NleD cut JNK1 within the activation loop (A).

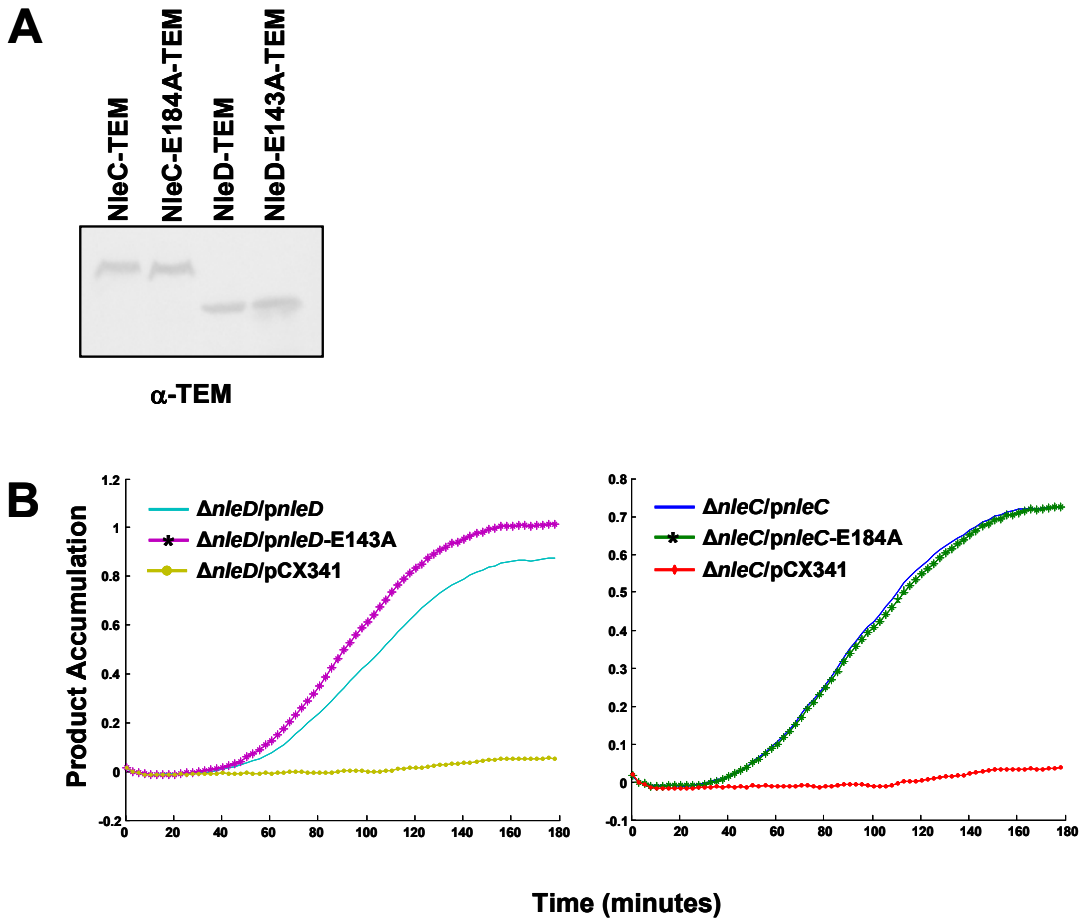

Fig. S6. NleC and NleD exhibit steady state levels in the bacteria and translocation efficiency, similar to those of the corresponding mutated proteins; NleC-E184A and NleD-E143A.

A. Proteins were extracted from bacteria containing pCX341 derivatives expressing NleC, NleD, NleC-E184A, or NleD-E143A, all C-terminally tagged with TEM. The levels of the proteins in the extracts were evaluated by Western blot using anti-TEM-1 antibody as described (Mills et al., 2008).

B. EPEC strains deleted of *nleD* or *nleC* and containing plasmids expressing TEM tagged NleC, NleD, NleC-E184A, or NleD-E143A, were used for translocation assay as described (Mills et al., 2008). The used strains are indicated within the graphs. This assay reflects the amount of translocated proteins inside the host cells and this amount is determined by both the efficiency of their translocation into the HeLa cells and their stability in the HeLa cells (Mills et al., 2008). As negative control we used EPEC containing the vector (pCX341) and expressing un-fused TEM-1.

The results in A and B show that the mutated NleC and NleD exhibit similar steady state levels and translocation efficiency to that of the wild type proteins.

MSDSKCDSQFYSVQVADSTFTVLKRYQQLKPIGSGAQGIVCAAFDTVLGINVAVKKLSRP
FQNQTHAKRAYRELVLLKCVNHKNIISLLNVFTPQKTLEEFQDVYLVMELMDANLCQVIH
MELDHERMSYLLYQMLCGIKHLHSAGIIHRDLKPSNIVVKSDCTLKILDFGLARTACTNF
MMTPYVVTRYYRAPEVILGMGYKENVDIWSVGCIMGELVKGCVIFQGTDHIDQWNKVIEQ
LGTPSAEFMKKLQPTVRNYVENRPKYPGIKFEELFPDWIFPSESERDKIKTSQARDLLSK
MLVIDPDKRISVDEALRHPYITVWYDPAEAEAPPPQIYDAQLEEREHAIEEWKELIYKEV
MDWEERSKNGVVKDQPSDAAVSSNATPSQSSSINDISSMSTEQTLASDTDSSLDASTGPL
EGCR

Fig. S7. JNK2 sequence

The sequence of JNK2 is shown. The activation loop that separates the N- and C- terminal domains of JNK2 is marked in red fonts and the TPY motif is framed. NleD cuts JNK within this motif between P and Y.

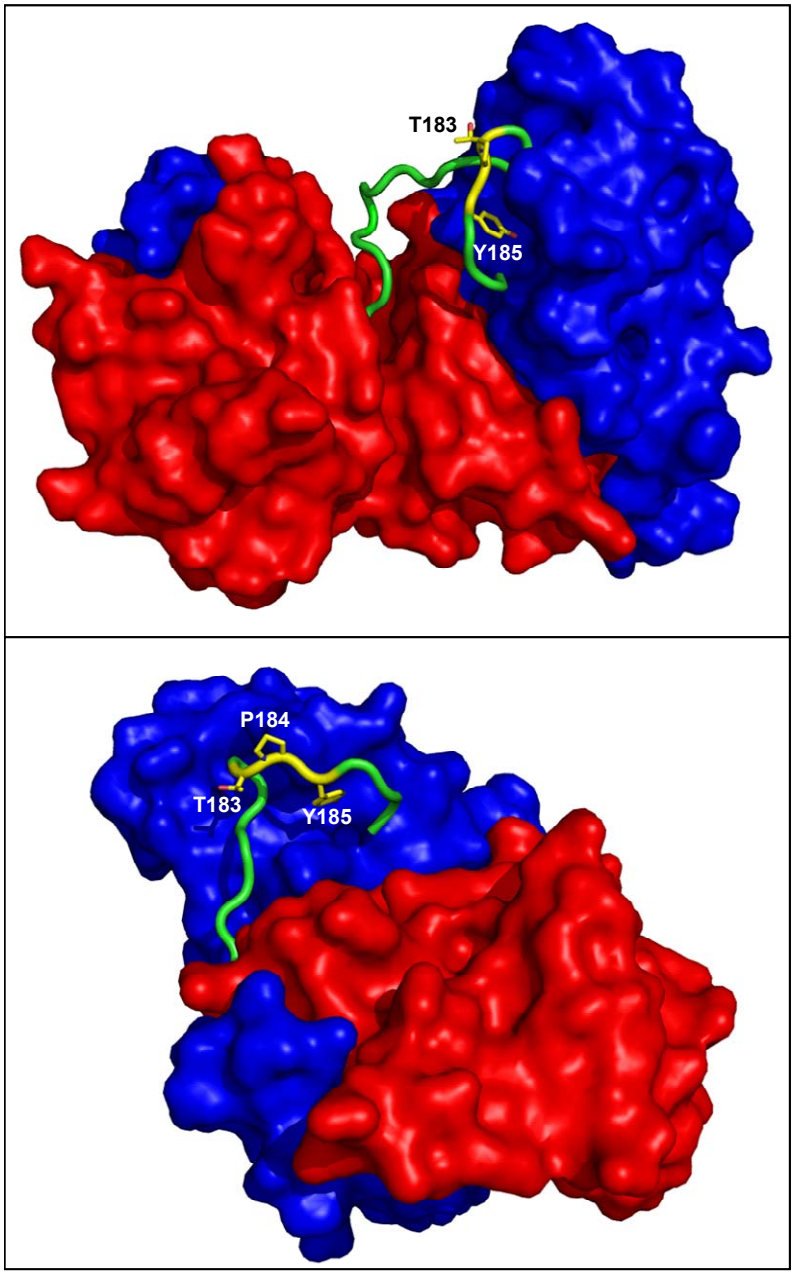

Fig. S8. Models of JNK2

Two views of JNK are shown. The N-terminal region is colored in red and the C-terminal in blue. The activation loop, which connects the two domains, is indicated in green and the TPY motif in yellow. NleD cuts between P184 and Y185.

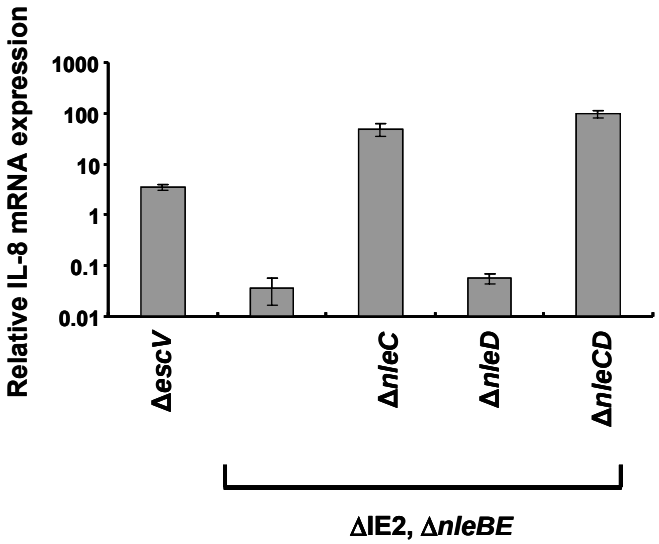

Fig. S9. NleD does not affect the transcription level of IL-8.

RKO cells were infected with different EPEC strains as indicated. The Δ*escV* (TTSS deficient strain) and ΔIE2, Δ*nleBE*, were used as positive and negative controls and the influence of further deletion of *nleC, nleD* or both was tested. To this end the cells were infected with the different EPEC strains for 3 h to allow injection of effectors before stimulation with TNFα for additional 3 h. Then RNA was extracted from the RKO cells and Real-time PCR performed to quantify IL-8 mRNA levels. Experiments were performed in triplicates and a typical experiment out of 2 is shown. Error bars indicate standard deviation.

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EPEC        ---------MKIPSLQSNFNFSAPAGYSAPIAPNRAENAYADYVLDIGKRIPLSAADLSN 51
EHEC        ---------MKIPSLQSNFNFSAPAGYSAPIAPNRAENAYADYVLDIGKRIPLSAADLSN 51
CR          ---------MKIPSLQPSFNFFAPAGYSAAVAPNRSDNAYADYVLDIGKRIPLSAEDLGN 51
YA          MYKIILGINMKITSLQPSFNFSAQEGYSAAVAPNHSDNAYADYVLDIGKRIPFSTADLGN 60
SE          ---------MKISSSTPCLNFAPQKEYSAAVVPHPSKNAYADYVLETGKRIPFSAADLSN 51
                     ***.*  . :** .   ***.:.*: :.********: *****:*: **.*

EPEC        VYESVIRAVHDSRSRLIDQHTVDMIGNTVLDALSRSQTFRDAVSYGIHNEKVHIGCIKYR 111
EHEC        VYESVIRAVHDSRSRLIDQHTVDMIGNTVLDALSRSQTFRDAVSYGIHNEKVHIGCIKYR 111
CR          LYENVIRAVRDSRSKLIDQHTVDMIGNTILDALSRSQTFRDAVSYGIHNKEVHIGCIKYR 111
YA          LYENVIHAVHDSLSRLIDQHTADMIGNTVLDALSRSQTFREAVSYGIHNNEVHLGCIKYR 120
SE          LYQSVIYAVHSSRSRLIDQHTANMIGNTVLDALSRSQTFRDAVIYGIHNKEVQLGCITYR 111
            :*:.** **:.* *:******.:*****:**********:** *****::*::***.**

EPEC        NEYELNEESSVKIDDIQSLTCNELYEYDVGQEPIFPICEAGENDNEEPYVSFSVAPDTDS 171
EHEC        NEYELNEESSVKIDDIQSLTCNELYEYDVGQEPIFPICEAGENDNEEPYVSFSVAPDTDS 171
CR          NEYELNGESPVKVDDIQSLTCTELYEYDVGQEPILPICEAGENDNEEPYVSFSVAPDTDS 171
YA          NEYELNGESPVEVDDIQSLTCTELYEYDVGQEPVFPICEAGEDDHEEPYVSFSVAPDADS 180
SE          NEYEINEDSPVGVDSIHLLTHSELYEYEAGQEPILPICEARKDEHEEAYISFSAAPDTDS 171
            ****:* :*.* :*.*: ** .*****:.****::***** ::::**.*:***.***:**

EPEC        YEMPSWQEGLIHEIIHHVTGSSDPSGDSNIELGPTEILARRVAQELGWSVPDFKGYAEPE 231
EHEC        YEMPSWQEGLIHEIIHHVTGSSDPSGDSNIELGPTEILARRVAQELGWSVPDFKGYAEPE 231
CR          YEMPSWQEGLIHEIIHHVTGASDPSGDSNIELGPTEILARRVAQELGWTVPDFIGYAEPD 231
YA          YEMPSWQEGLIHEIIHHVTGASDPSRDSNIELGPTEILARRVAQELGWPIPDFIGYADPD 240
SE          CEMPSWQEGLIHEIIHHVTGAGDPLEDGNIEPGPTEILARRIAQELGWSIPEFTGYASPD 231
             ******************:.**  *.*** ********:******.:*:* ***.*:

EPEC        REAHLRLRNLNALRQAAMRHEENERAFFERLGTISDRYEASPDFTEYSAVSNIGYGFIQQ 291
EHEC        REAHLRLRNLNALRQAAMRHEENERAFFERLGTISDRYEASPDFTEYSAVSNIGYGFIQQ 291
CR          REAHLRGRNLNALRQAAMRHEDNERTFFERLGMISDRYEASPDFTEYSAVSNIEYGFIQQ 291
YA          REAHLRARNLNALRQAATRHESNEGAFFERLDMISDKYEVSPDFTEYAVVSNIEYGLIQQ 300
SE          RVAHLRTRNLNALRQTATRHEDNEEAFFERLDVISEGYEASADFTEYPVMSDMVKELNKP 291
            * **** ********:* ***.** :*****. **: **.*.*****..:*::   : :

EPEC        HDFPGLAINDNLQDAN--QIQLYHGAPYIFTFGDVDKHNQR 330
EHEC        HDFPGLAINDNLQDAN--QIQLYHGAPYIFTFGDVDKHNQQ 330
CR          HDFPGLAIDDNLQDAN--QIQLYHGAPYIFTFGDVDKHNQR 330
YA          HDFPGLAIDDNLQDAN--QIQLYHGAPYIFTFGGADRHNQR 339
SE          HDFPGLVINDNTMDADPDQIQLYHGQPYIFTF--VDKHNQR 330
            ******.*:**  **:  ******* ******  .*:***:
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Fig. S10. Multiple alignment of NleC homologs

The NleC protein sequences of EPEC (*Escherichia coli* O127:H6 str. E2348/69), EHEC (*Escherichia coli* O157:H7 EDL933), CR (*Citrobacter rodentium* ICC168), YA (*Yersinia aldovae*) and SE (*Salmonella enterica* subsp. enterica serovar Javiana) were aligned using ClustalW2. The HExxH Zn-metalloprotease motif is highlighted.

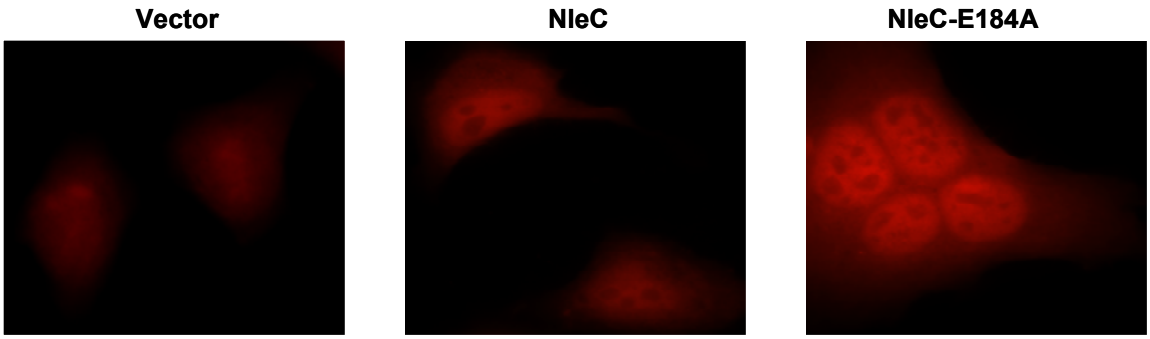


Fig. S11. Localization of transiently expressed mCherry-NleC

HeLa cells were transfected with plasmid expression mCherry (Vector), mCherry-NleC (NleC) or mCherry-NleC-E184A (NleC-E184A). After 16 h cells were fixed and analyzed by microscopy. The mCherry-NleC proteins exhibit distribution different from that of un-fused mCherry. mCherry was distributed in the cells with some enhancement in the nuclear region and the mCherry-NleC proteins were localized predominantly to the nucleus. The appearance of mCherry and mCherry-NleC proteins in the nuclear region exhibit very different appearance. The former appear as diffuse staining with some enhancement in the nuclear region, but without define borders. In contrast the mCherry-NleC proteins staining were clearly framed in the nucleus although some cytoplasmic staining was also evident. In addition, only the mCherry-NleC staining emphasized the location of the nucleoli as darker region in the nucleus, which was contrasted against the mCherry-NleC staining.

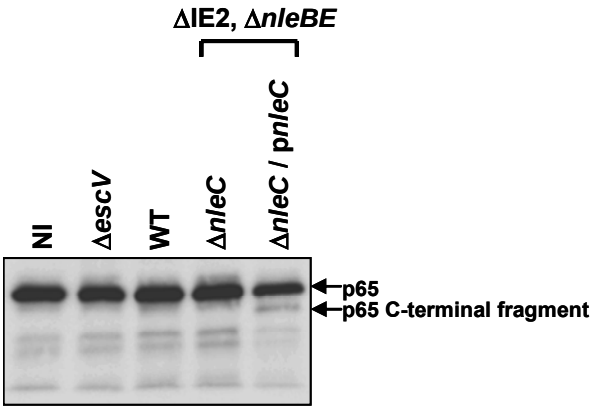


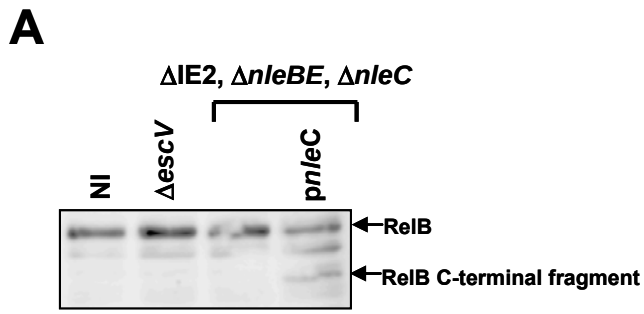
Fig. S12. NleC cleave p65 *in vivo* in Caco2 cells

Caco2 cells were infected for 3 h with different EPEC strains as indicated above the lanes, or remained non infected (NI). Cytoplasmic proteins were then extracted and subjected to Western blot analysis with anti-p65 antibody. The locations of the intact and fragmented p65 proteins are indicated. Mutant with inactivated TTSS (Δ*escV*) was used as negative control.

MDELFPLIFPAEPAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERSTDTTKTHPT
IKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFYEAELCPDRCIHSFQNLGIQC
VKKRDLEQAISQRIQTNNNPFQVPIEEQRGDYDLNAVRLCFQVTVRDPSGRPLRLPPVLP
HPIFDNRAPNTAELKICRVNRNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPGWEARGSFS
QADVHRQVAIVFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDTDDRHRIEE
KRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQPYPFTSSLSTINY
DEFPTMVFPSGQISQASALAPAPPQVLPQAPAPAPAPAMVSALAQAPAPVPVLAPGPPQA
VAPPAPKPTQAGEGTLSEALLQLQFDDEDLGALLGNSTDPAVFTDLASVDNSEFQQLLNQ
GIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLGAPGLPNGLLSGDEDFSSIADM
DFSALLSQISS

Fig. S13. The p65 sequence

The sequence of p65 is shown. The REL domain is underlined and residues that directly contact the DNA and known to be important for DNA binding are marked in red fonts. NleC cleaves p65 between the framed C and E.



B

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RelB    134   KQRGMPFRYECEGRSAGSILGESSTE   159
p65      28   KQRGMRFRYKCEGRSAGSIPGERSTD    53
              ***** ***:********* ** **:
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Fig. S14. NleC injection is associated with cleavage of RelB

A. HeLa cells were infected for 3 h with different EPEC strains as indicated above the lanes. Proteins were then extracted and subjected to Western blot analysis with anti-RelB antibody. The locations of the intact and fragmented RelB proteins are indicated. Expression of NleC correlated with RelB cleavage (in the right lane).

B. Comparison between the p65 cleavage site and the corresponding region in RelB. NleC cleave p65 between the framed C and E. This region is highly conserved and based on the size of the fragmented RelB it is possible that NleC cleaves RelB at the same site.

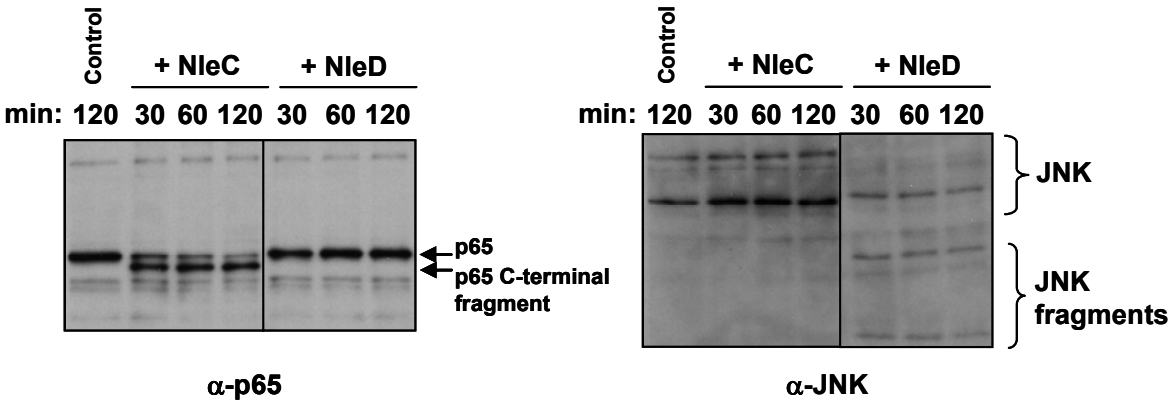

Fig. S15. NleC and NleD exhibit substrate specificity.

Cytosolic fraction of HeLa extracts was treated for different periods of time with purified NleC or NleD or remained untreated (Control), as indicated above the lanes. Reaction was stopped by boiling in SDS loading buffer, and products were visualized by Western blot analysis using anti-p65 and anti-JNK antibodies. Intact and clipped p65 and JNK are indicated.

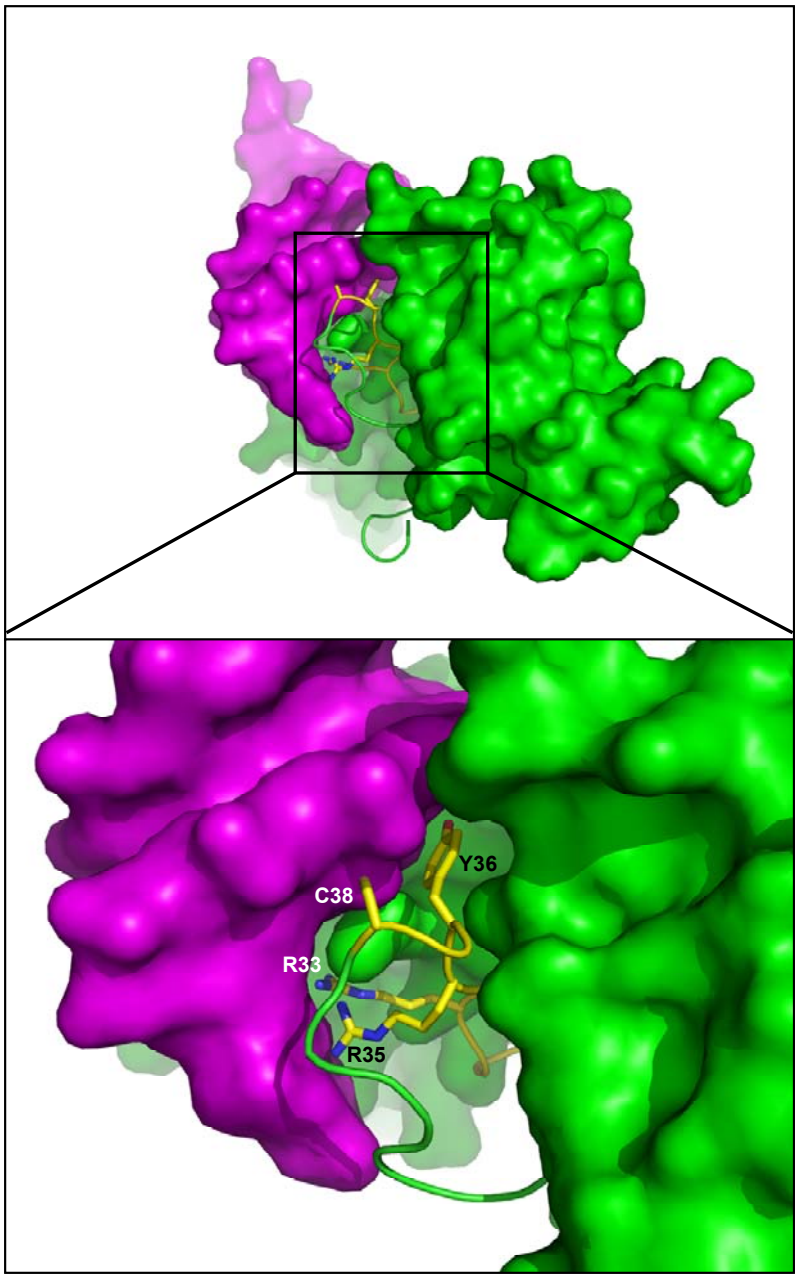

Fig. S16. A model of p65-DNA interaction

A model of p65 (green) bound to DNA (magenta) is shown (Chen et al., 1998). The clipped N-terminal region is colored in yellow. Residues that directly contact the DNA (R33, R35, Y36 and C38) are highlighted and marked. NleC cut p65 between residues C38 and E39.

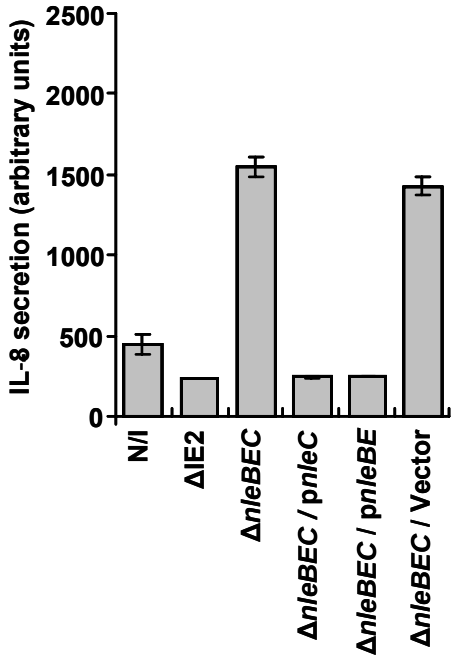

Fig. S17. Over expression of either NleC or NleBE by the infecting EPEC is sufficient to achieve full inhibition of IL-8 secretion.

HeLa cells were infected with ΔIE2 mutant EPEC, which was used here as wild type, or with a triple mutant Δ*nleBE,* Δ*nleC* (Δ*nleBEC*), or with the triple mutant complemented with plasmids expressing NleC, NleBE or vector (p*nleC*, p*nleBE*, and Vector, respectively). Uninfected and untreated cells served as the positive control (no IL-8 secretion –full repression) and uninfected cells stimulated with TNFα as the negative control (high IL-8 secretion-no repression), respectively. HeLa cells were infected with the relevant EPEC for 3 h to allow injection of effectors before stimulation with TNFα for 16 h. Finally, the growth media was harvested and the amount of secreted IL-8 determined using the ELISA assay.

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