nature microbiology

SUPPLEMENTARY INFORMATION

ARTICLE NUMBER: 16188 | DOI: 10.1038/NMICROBIOL.2016.188

Helicobacter pylori exploits human CEACAMs via HopQ for adherence and translocation of CagA

Verena Königer[1*], Lea Holsten[1*], Ute Harrison[1], Benjamin Busch[1], Eva Loell[1], Qing Zhao[1], Daniel A. Bonsor[7], Alexandra Roth[2], Arnaud Kengmo-Tchoupa[2], Stella I. Smith[4], Susanna Mueller[5], Eric J. Sundberg[8,9], Wolfgang Zimmermann[6,7], Wolfgang Fischer[1], Christof R. Hauck[2] and Rainer Haas[1,3§]

[1]Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Ludwig-Maximilians-Universität, Pettenkoferstraße 9a, D-80336 München, Germany

[2]Lehrstuhl Zellbiologie, Fachbereich Biologie, Universität Konstanz, Germany

[3]German Center for Infection Research (DZIF), partner site LMU München, Germany.

[4]Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, 6 Edmond Crescent, Yaba, Lagos, Nigeria

[5]Institut für Pathologie, Ludwig-Maximilians-Universität, München, Germany

[6]Tumor Immunology Laboratory, LIFE Center, Ludwig-Maximilians-Universität, München, Germany

[7]Department of Urology, University Hospital, Munich, Germany

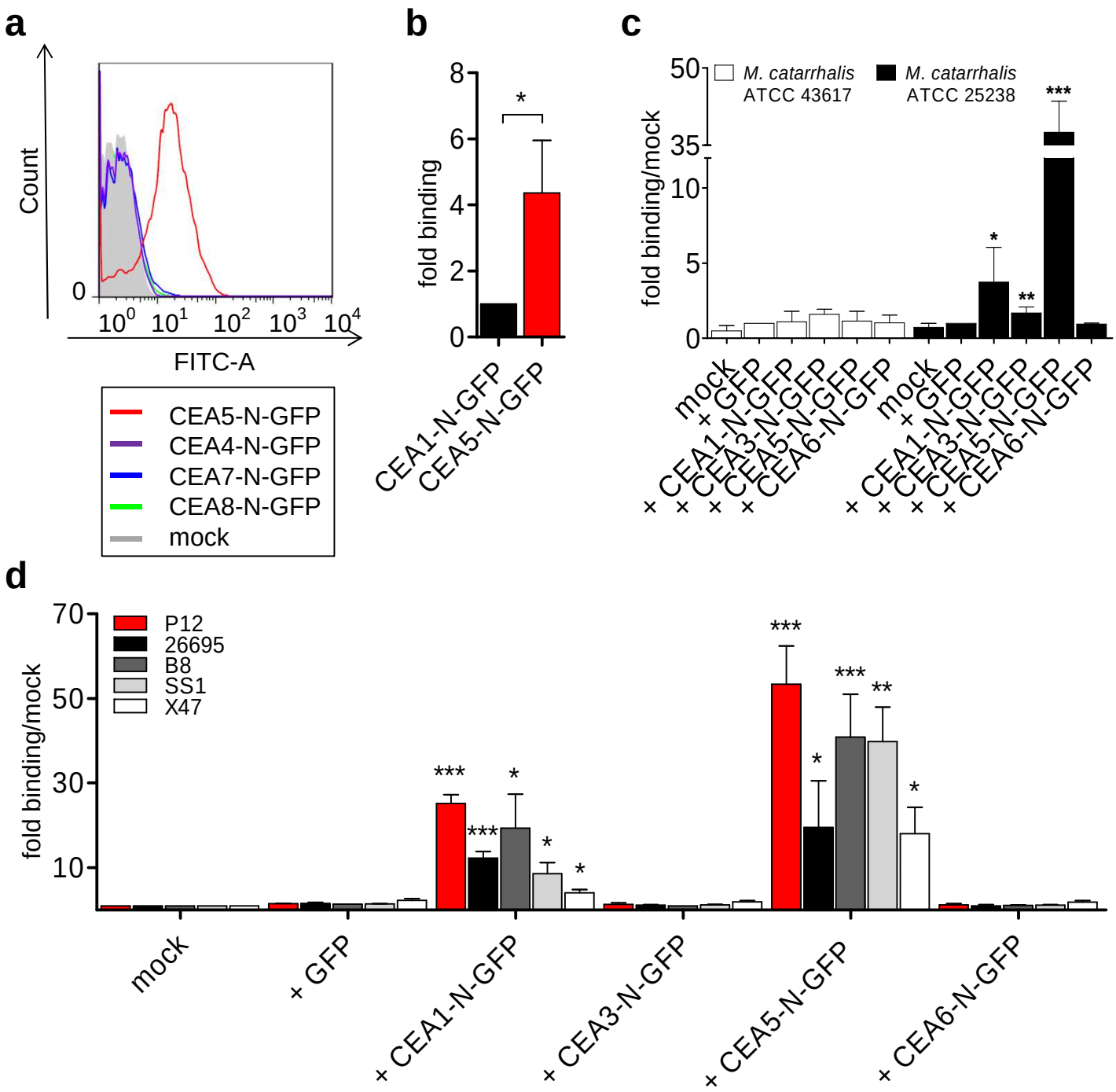
[8]Institute of Human Virology, University of Maryland School of Medicine, University of Maryland, Baltimore, Maryland 21201

[9]Departments of Medicine and Microbiology and Immunology, University of Maryland School of Medicine, University of Maryland, Baltimore, Maryland 21201

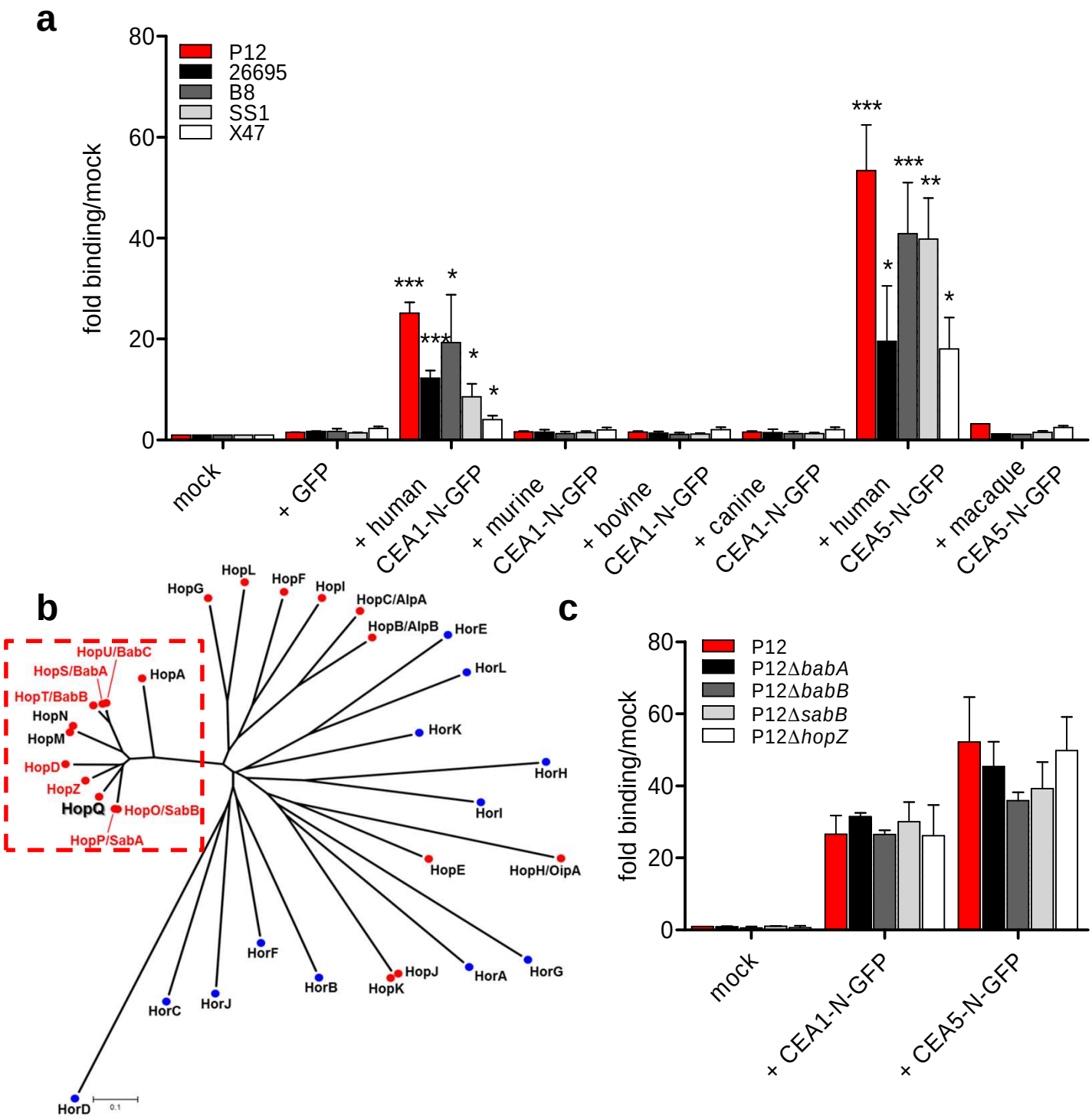
*These authors contributed equally to this work.

§ Corresponding author

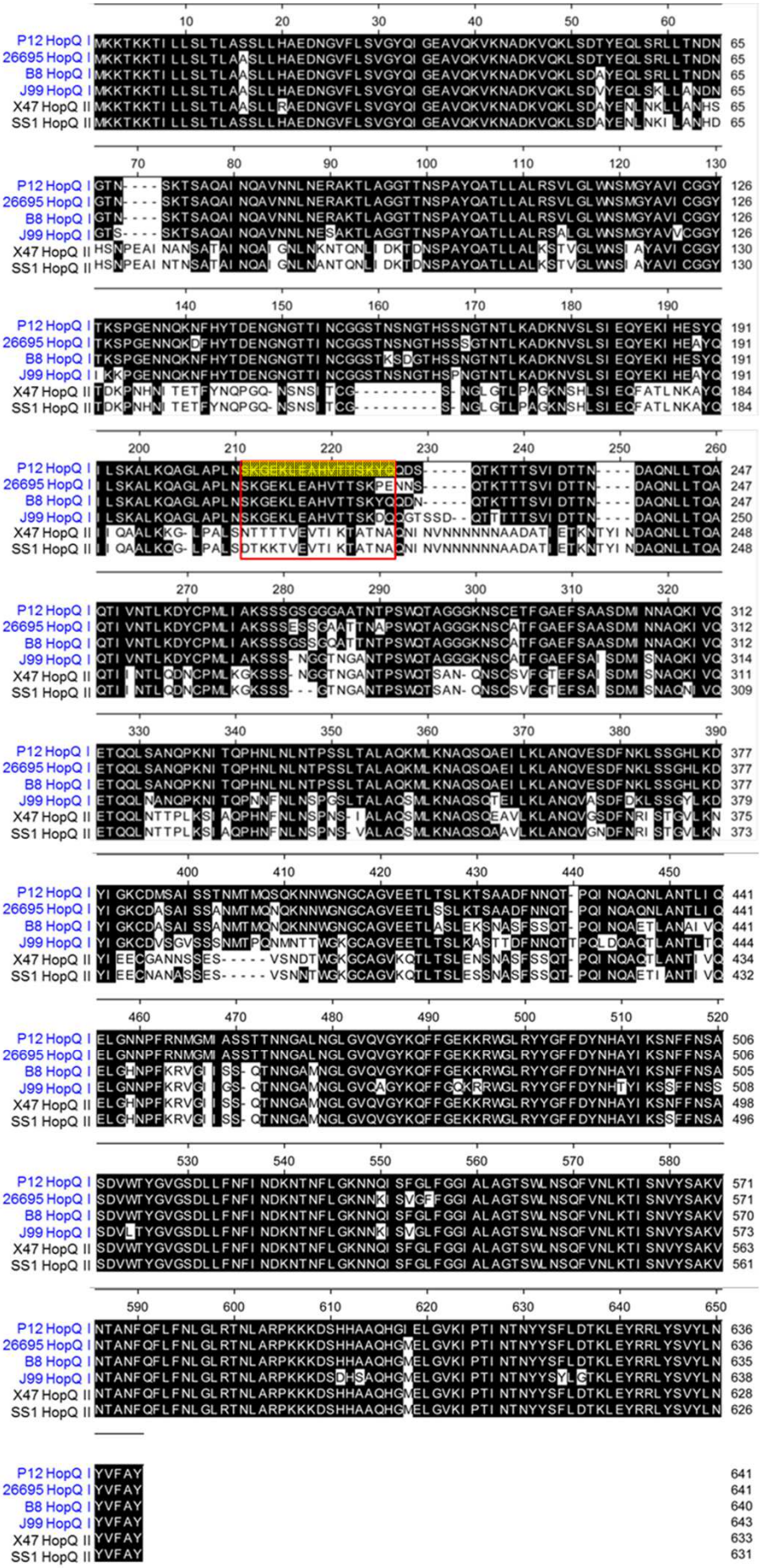
This PDF file includes:
Supplementary Figures 1 to 10
Supplementary Table 1 to 4



Supplementary Figure 1. Bacterial pulldown assays to determine binding of different soluble CEA-N-GFP constructs to *Moraxella catarrhalis*, *Hp* P12 and other *Hp* strains.
a, The *Hp* P12 strain was incubated with different CEA-N-GFP culture supernatants (CEA4-N-GFP, CEA5-N-GFP, CEA7-N-GFP, CEA8-N-GFP, GFP, control). Only CEA5-N-GFP interacted with *Hp* P12. This is a representative flow cytometry plot of an experiment that was performed three times. **b,** Soluble CEA5-N-GFP binds 4.3 x more to *Hp* P12 as compared to CEA1-N-GFP. **c,** *M. catarrhalis strain* ATCC 43617, (negative control) and strain 25238 (positive for CEACAM binding) were incubated with different CEA-N-GFP culture supernatants (CEA1-N-GFP, CEA3-N-GFP, CEA5-N-GFP and CEA6-N-GFP) or GFP alone. **d,** Different *Hp* strains (human, Mongolian gerbil-adapted, mouse-adapted) were incubated with CEA-N-GFP culture supernatants. Binding was only found for CEA1-N-GFP and CEA5-N-GFP, although to a varying degree. Student's t-test, one-tailed; *p<0.05), **p<0.01), ***p<0.001). Values are means +/- SEM.

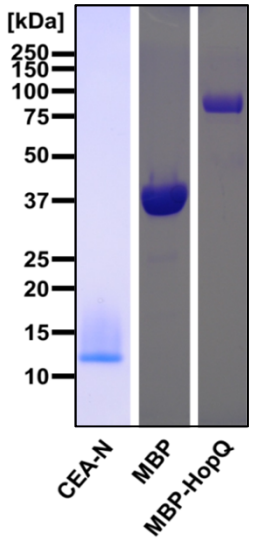


Supplementary Figure 2. Binding of *Hp* wt or isogenic adhesin mutants to human or non-human CEA-N-GFP proteins and the putative adhesin branch of the large Hop and Hor outer membrane protein family. a, *Hp* strains (human, Mongolian gerbil-adapted, mouse-adapted) were incubated with CEA-N-GFP culture supernatants of the indicated mammalian CEACAMs or GFP-transfected cells (neg. control) **b,** The phylogenetic tree was constructed and tested by neighbor joining with MEGA5.2. Hop proteins are shown in red, Hor proteins in blue. The putative adhesin branch (red dotted line box) contains most of the so far identified *Hp* adhesins (underlined). **c**, Soluble CEA1-N-GFP and CEA5-N-GFP constructs, or a mock control, were incubated with *Hp* strains P12, and a set of isogenic mutants in *Hp hop* genes, such as P12Δ*babA*, P12Δ*babB*, P12Δ*sabB*, P12Δ*hopZ* and the binding capacity to CEACAMs was tested. Student's t-test, two-tailed; *p<0.05), **p<0.01), ***p<0.001). Values are means +/- SEM. Experiments were performed three times with similar results.

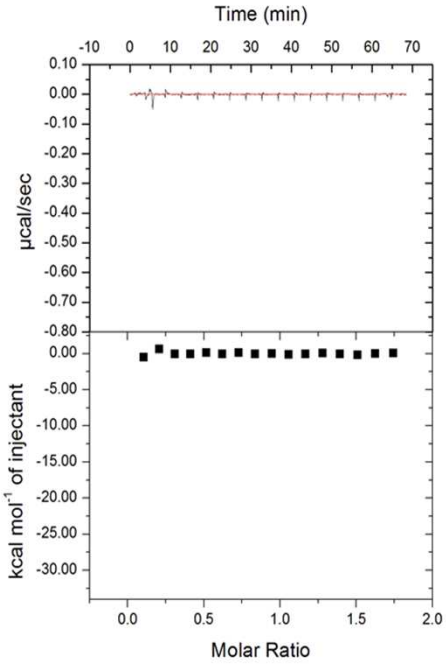


Supplementary Figure 3. Amino acid sequence comparison of HopQ type I and HopQ type II.
Amino acid sequences of different HopQ proteins were compared using the MegAlign Software for Multiple Sequence Alignments (DNA Star Lasergene, Madison, USA) with $HopQ_{P12}$ as reference. Identical amino acids are marked by black shading. Please note that HopQ I proteins (blue text) are relatively conserved to each other, whereas HopQ II sequences differ substantially (black text). The peptide sequence chosen for generation of a HopQ I antiserum is taken from strain P12 (yellow sequence). The red box highlights the conservation of HopQ I sequences recognized by the anti-HopQ I peptide antibody (AK298) and shows that HopQ II proteins are highly divergent and therefore not recognized. $HopQ_{26695}$ and $HopQ_{J99}$ show a sequence divergence in the antibody recognition sequence (Y225P, Q226E and Y225D, respectively), which results in a weaker recognition of the proteins by AK298.

a

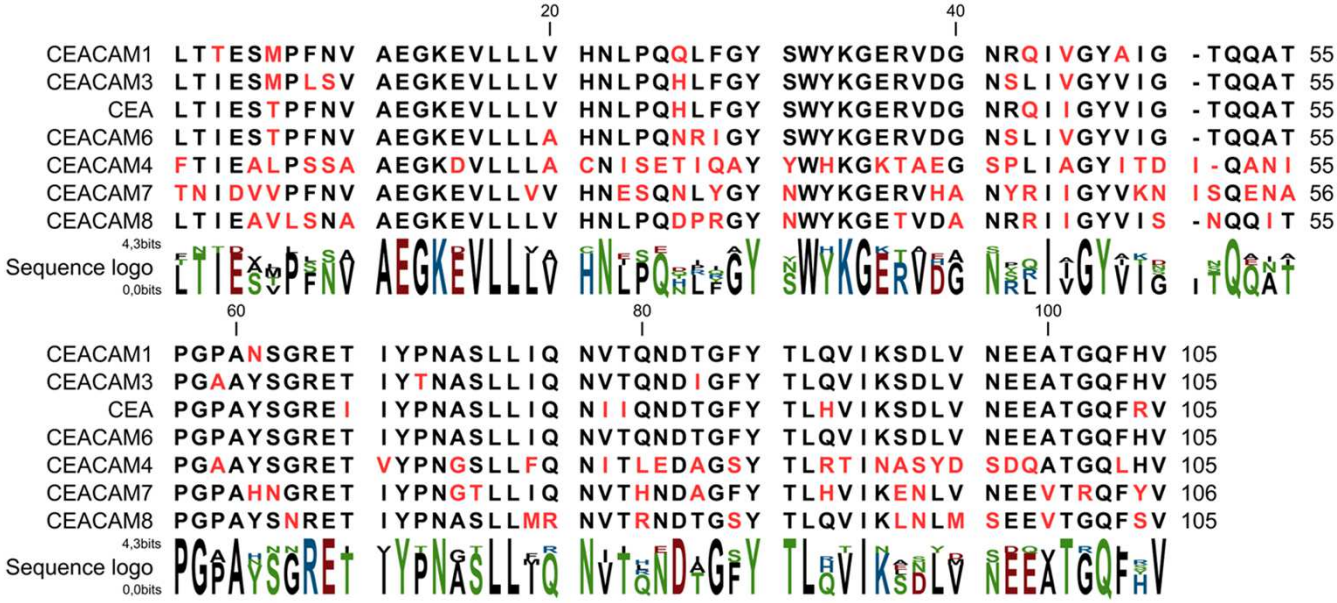


b

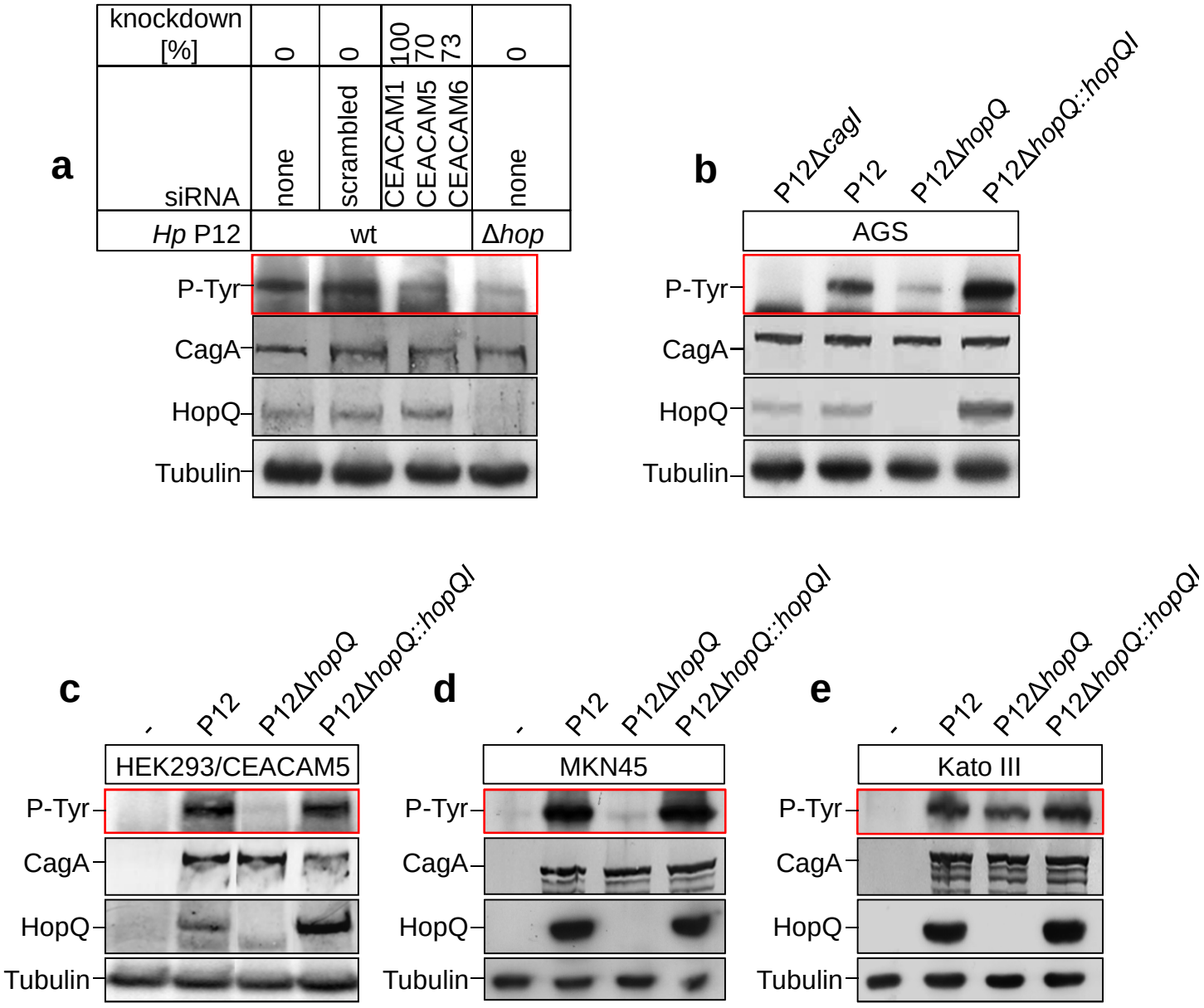


MBP/CEA-N

c

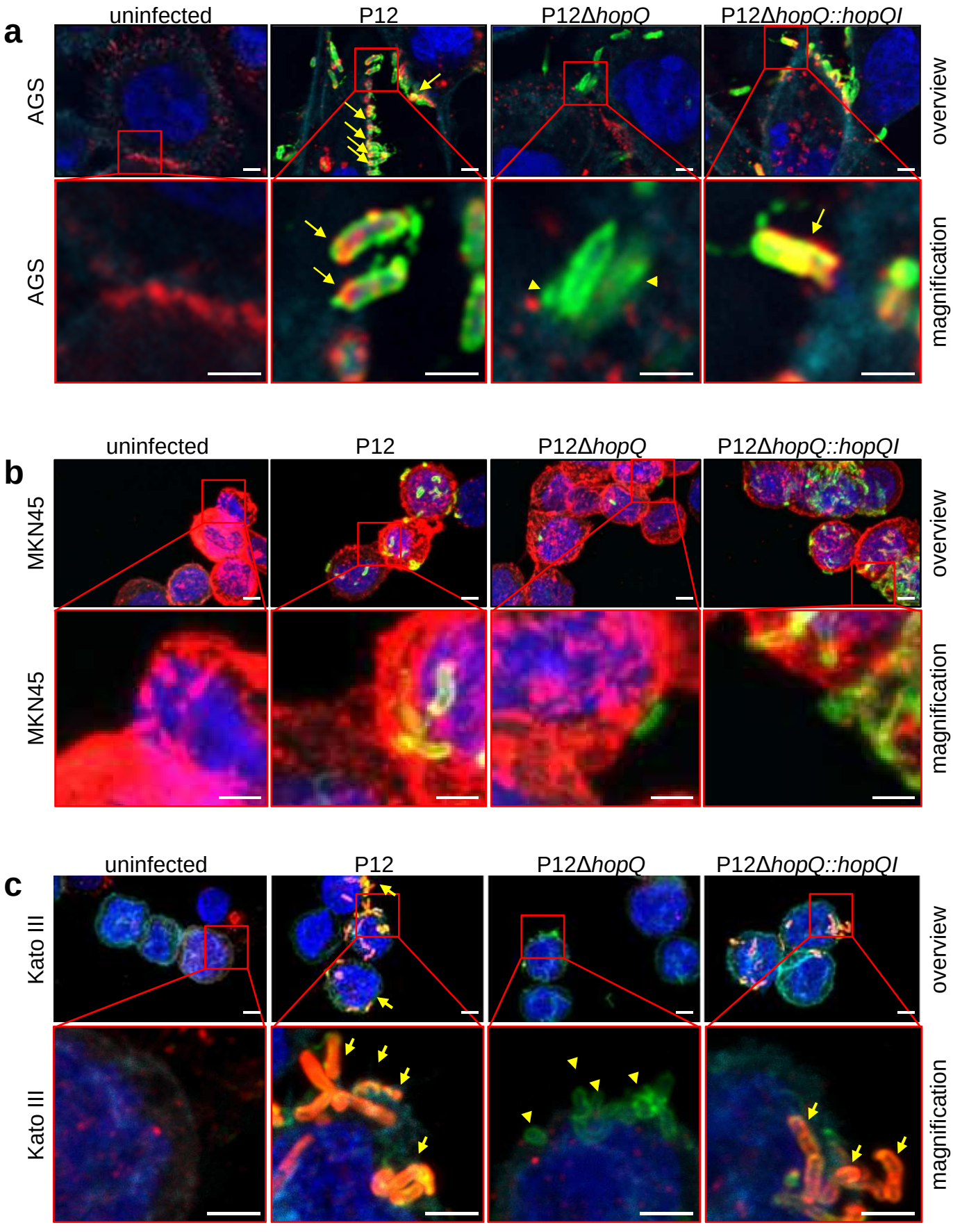


Supplementary Figure 4. Generation and purification of MBP fusion proteins and ITC experiments. **a**, Coomassie-stained SDS polyacrylamide gel showing the purified CEA-N, MBP and MBP-HopQ proteins. **b,** Isothermal titration calorimetry binding curve of the CEACAM5 N-terminal IgV-like domain (CEA-N) titrated against MBP alone. **c,** Multiple alignment of CEACAM1, CEACAM3, CEACAM4, CEACAM5 (CEA) CEACAM6, CEACAM7 and CEACAM8 N-terminal domains, generated by CLC Workbench 6. Conserved amino acids are shown in black, variable ones in red. A consensus sequence is shown below.

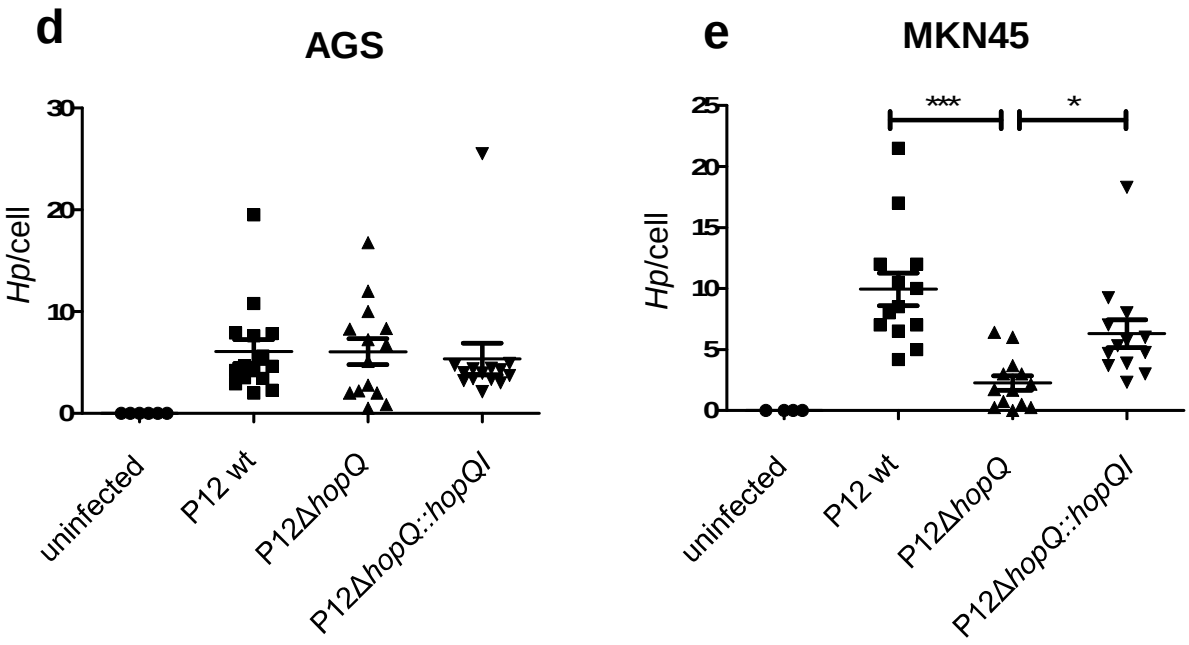


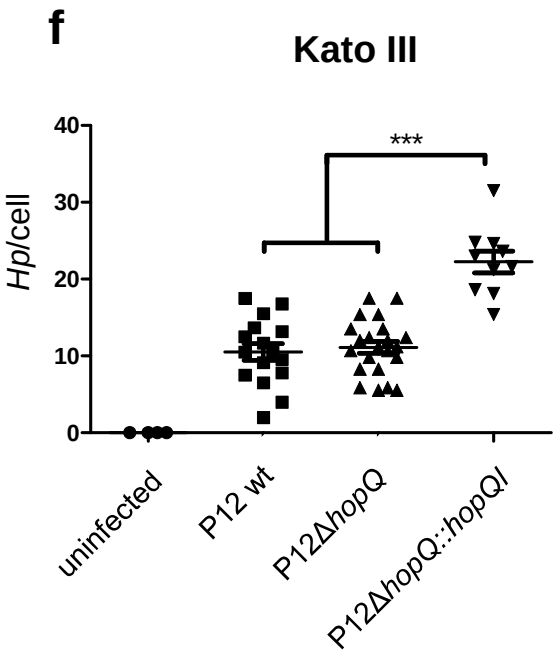
Supplementary Figure 5. CagA translocation efficiency correlates with the adherence of *Hp* to gastric cells.

a, CEACAM1 (100%), CEACAM5 (70%) and CEACAM6 (73%) knockdown as measured by flow cytometry with corresponding antibodies. Infection experiments with *Hp* P12 or P12Δ*hopQ* were performed. CagA translocation in CEACAM knockdown cells was reduced to a level similar to that of a P12Δ*hopQ* mutant strain. **b**-**e**, Western blots showing CagA translocation of P12 wt, P12Δ*hopQ* mutant and the genetically complemented strain (P12Δ*hopQ::hopQI)* into AGS (**b**), HEK293/CEACAM5 stable transfected cells (**c**), into MKN45 (**d**) and Kato III gastric epithelial cells (**e**). Antibodies 4G10 (P-Tyr), AK268 (CagA), AK298 (HopQI) and anti-tubulin were used. Representative western blots of an experiment that was performed three times are shown.

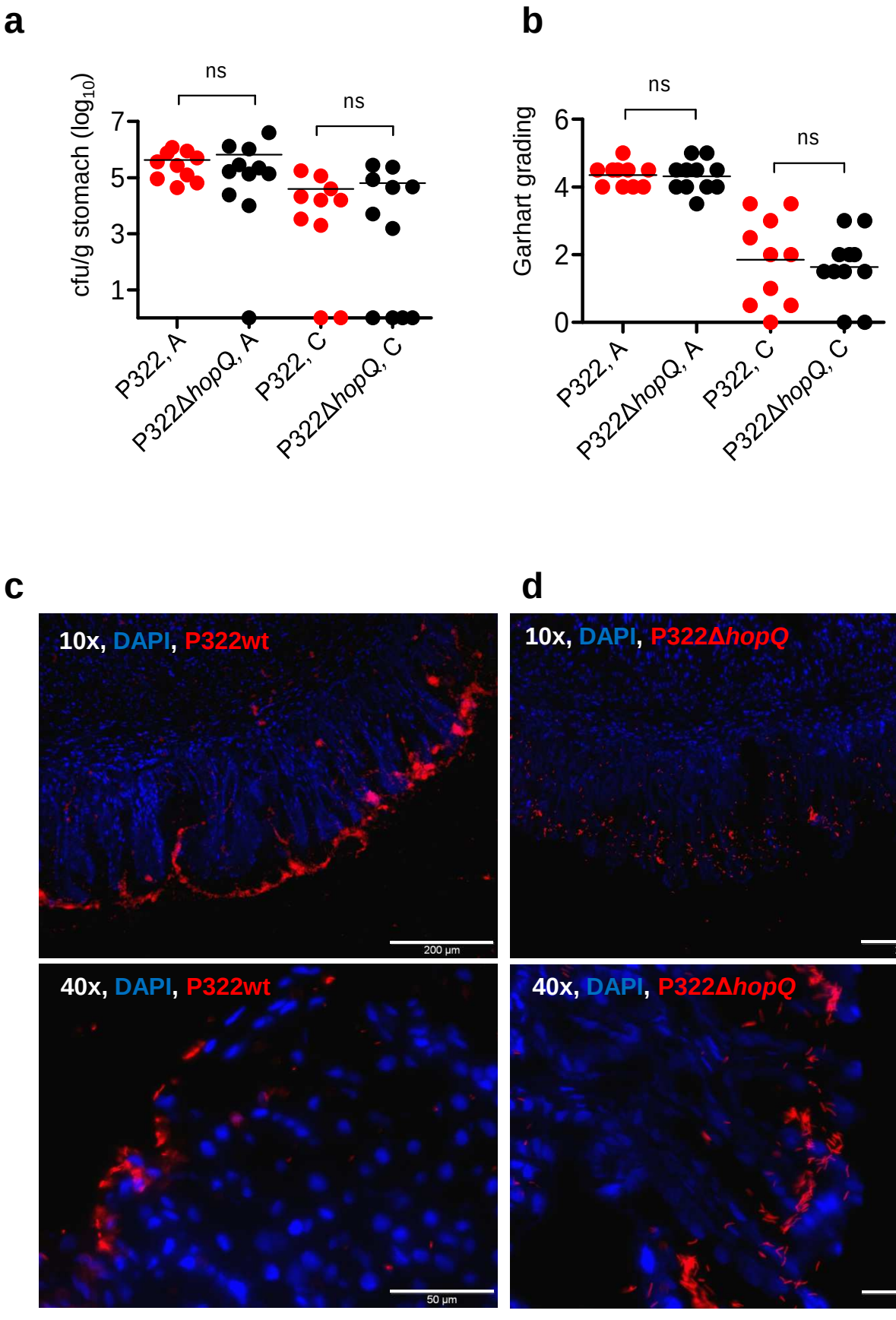


Supplementary Figure 5, continuation next page

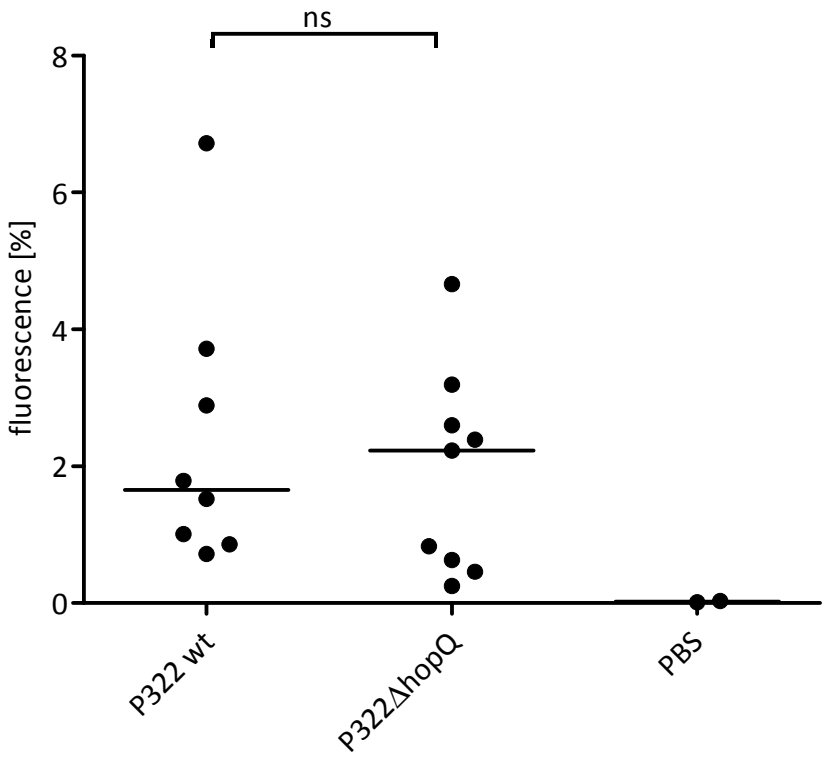




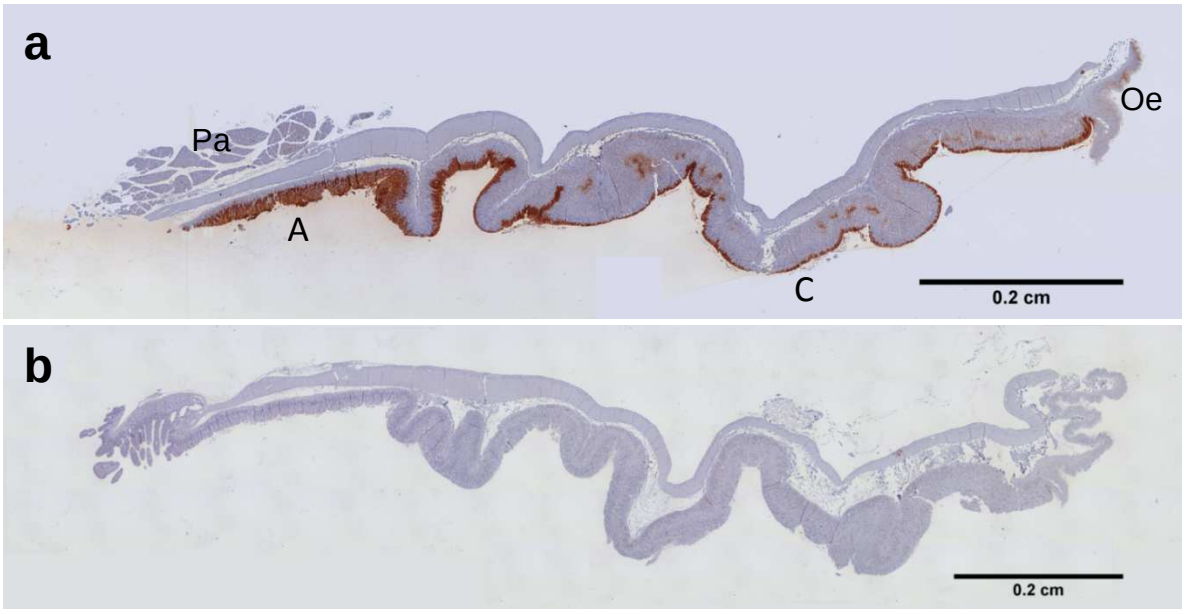
Supplementary Figure 6. Confocal Laser Scanning Microscopy (CLSM) studies of epithelial cell lines AGS, KatoIII and MKN45 infected with *Hp* P12 wt and mutant strains. a, Confluent monolayers of AGS (**a**), MKN45 (**b**) and Kato III (**c**) cells were infected with *Hp* P12 wt, P12Δ*hopQ* or P12Δ*hopQ::hopQI*. The cells were analysed by CSLM (63 x objective) by a Leica SP5 microscope. The top row shows an overview, the lower row a magnification of characteristic observation fields. Several planes are combined to obtain a Z-stack (bottom to top), showing bacterial binding and colocalization of CEACAM5 and *Hp*. Yellow arrows point to co-localisation events between *Hp* and CEACAM, arrowheads point to the *hopQ* strain showing binding, but no co-localization with CEACAM5. Scale bars represent 2.5 μm. At least 3 micrographs of independent cell culture samples were taken, one representative area is shown. To obtain quantitative data the numbers of *Hp*/cell were counted for each cell line and presented as graphs (**d-f**). Statistics: One-way Anova (Tukey's post hoc test)
*: p<0.5, **: p<0.1, ***: p<0.01.

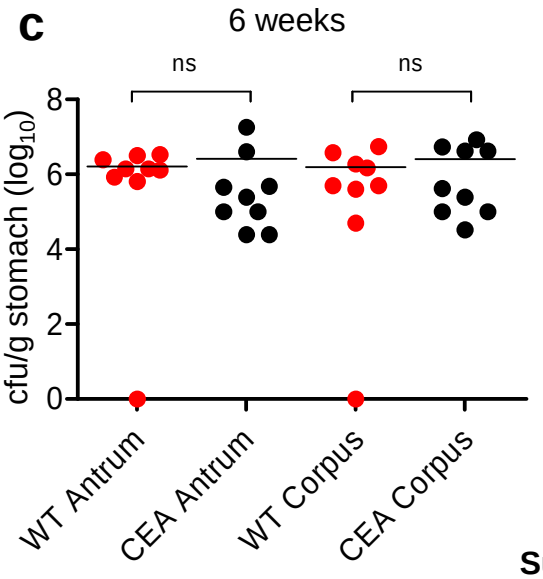

a
b
c
d
ns
ns
cfu/g stomach (log10)
7
5
3
1
P322, A
P322ΔhopQ, A
P322, C
P322ΔhopQ, C
Garhart grading
6
4
2
0
10x, DAPI, P322wt
10x, DAPI, P322ΔhopQ
40x, DAPI, P322wt
40x, DAPI, P322ΔhopQ
200 µm
50 µm

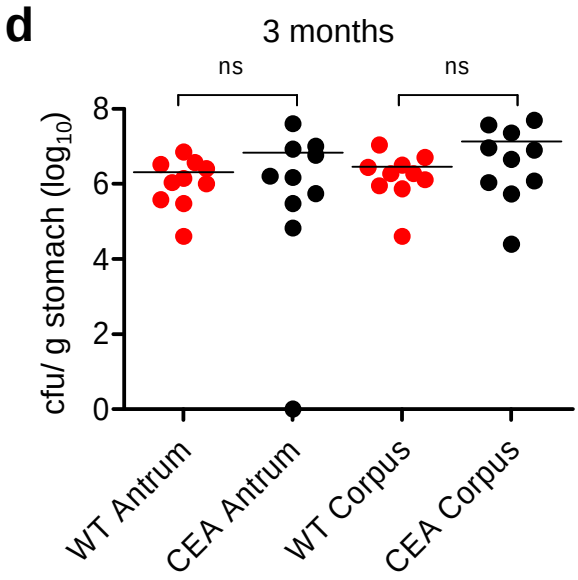
e

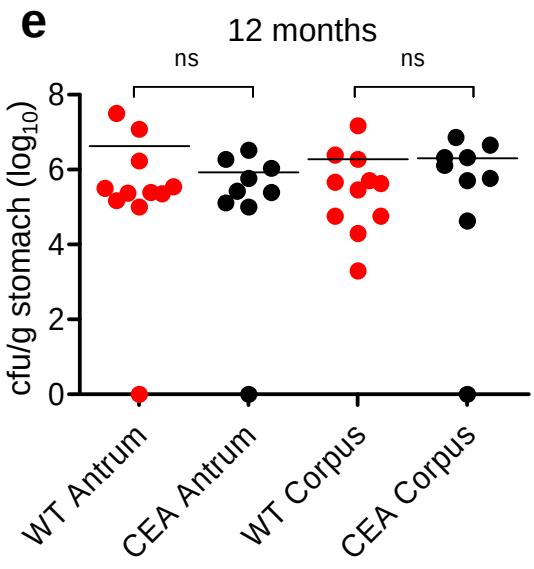


Supplementary Figure 7. Mongolian gerbil infection of *Hp* P322 and P322Δ*hopQ* mutant and histological grading of antral and corpus mucosa. **a,** Mongolian gerbils were orally infected for 8 weeks with the Mongolian gerbil-adapted strain *Hp* P322 wt (red dots), or the isogenic mutant P322Δ*hopQ* (black dots). Colony forming units (CFU/g) were determined by plating antrum (A) and corpus (C) stomach material. **b**, Grading of histopathological changes was performed according to Garhart *et al*. [28]. Statistics: Mann-Whitney-U-test, two-tailed; ns, not significant. Values are means. *Hp* P322, n=9; P322*ΔhopQ*, n=11. **c**, **d,** Gastric cryo-sections of the Mongolian gerbil stomach were incubated with Tetramethylrhodamine (TAMRA)-labelled *Hp* strain P322 or the isogenic mutant P322*ΔhopQ* (red fluorescent). After extensive washing of unbound bacteria micrographs of each gastric section were taken (10x and 40x objective). One representative area is presented. Nuclei are stained by DAPI (blue). Size of scale bars is depicted (50 µm or 200 µm). **e,** The bacterial binding was quantified by determining the red fluorescence (percentage fluorescence in a size-matched tissue area (see online methods section for details). Biological replicates, n=6 (P322 wt), 9 (P322 *ΔhopQ* and n=2, PBS. Mann-Whitney-*U*-test for unpaired groups; ns, not significant. Values are means +/- SEM.

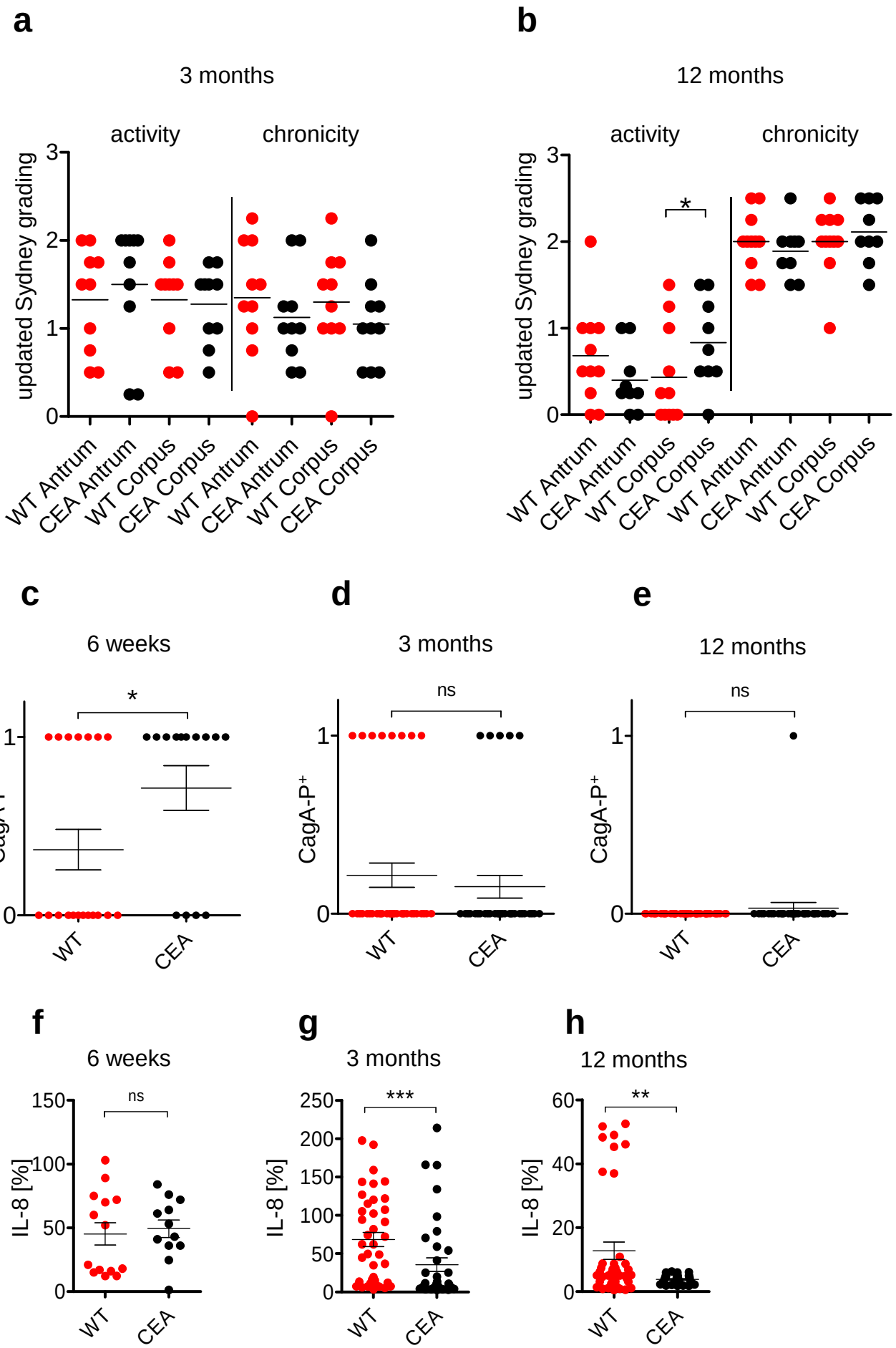






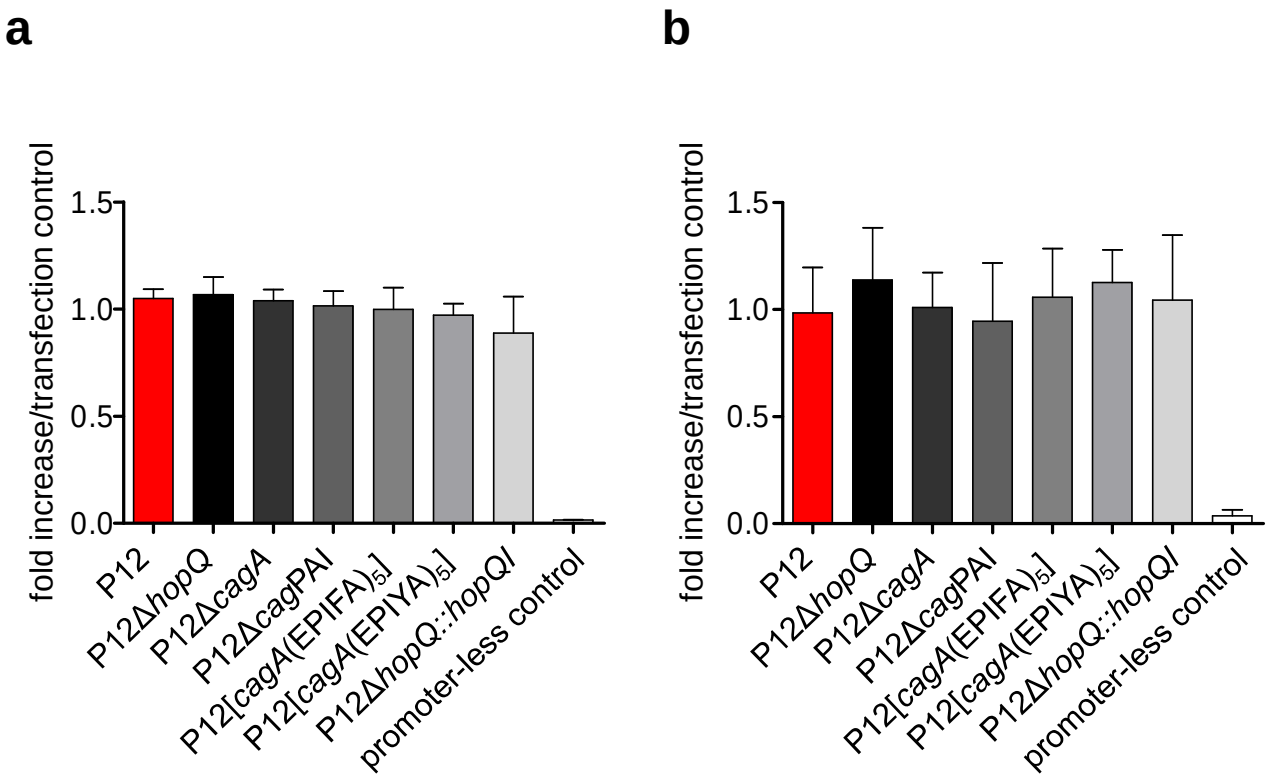


Supplementary Figure 8. The CEA mouse model. a-b, Expression of CEACAM5 in gastric mucosa of humanized CEA-transgenic mice. Immunohistological detection of CEACAM5 in longitudinal section of paraffin-embedded stomach tissue of a 24 weeks old CEA (line CEA2682) mouse (**a**) and a 29 weeks old non-transgenic littermate (WT) (**b**). A, antrum; C, corpus; Oe, oesophagus; P, pancreas. n=2, one representative micrograph is shown. **c-e**, mice were orally infected with the mouse-adapted *Hp* PMSS1 strain for 6 weeks (**c**), 3 months (**d**), or 12 months (**e**). Colony forming units (CFU/g) were determined by plating antrum and corpus stomach material. Mann-Whitney-*U*-test for unpaired groups; ns, not significant. Values are means +/- SEM.


a
3 months
activity
chronicity
updated Sydney grading
0
1
2
3
WT Antrum
CEA Antrum
WT Corpus
CEA Corpus
WT Antrum
CEA Antrum
WT Corpus
CEA Corpus
b
12 months
activity
chronicity
updated Sydney grading
*
0
1
2
3
WT Antrum
CEA Antrum
WT Corpus
CEA Corpus
WT Antrum
CEA Antrum
WT Corpus
CEA Corpus
c
6 weeks
*
CagA-P+
0
1
WT
CEA
d
3 months
ns
CagA-P+
0
1
WT
CEA
e
12 months
ns
CagA-P+
0
1
WT
CEA
f
6 weeks
ns
IL-8 [%]
0
50
100
150
WT
CEA
g
3 months
***
IL-8 [%]
0
50
100
150
200
250
WT
CEA
h
12 months
**
IL-8 [%]
0
20
40
60
WT
CEA

Supplementary Figure 9. The CEA mouse differs in its response to *Hp* infetions as compared to the WT.

a-b, grading of histopathological changes of the stomach sections was performed for activity and chronicity on WT or CEA mice infected for 3 months (**a**) or in infections for 12 months (**b**). The interpolated lines show the means of the respective groups. n = 9-11 **c-e**, CagA translocation capacity of *Hp* reisolates after 6 weeks (**c**), 3 months (**d**) or 12 months (**e**) of infection (0: translocation-deficient, 1: translocation-competent) **f-h**, IL-8 induction capabilities on AGS cells of *Hp* reisolates from infected WT or CEA mice after 6 weeks (**f**), 3 months (**g**) or 12 months (**h**) of infection. Experiments (Western blots, IL-8 ELISA) were performed three times with similar results. Statistics: **a,b,f-h,** Mann-Whitney-*U*-test for unpaired groups, **c-e**, Chi-square test ; ns, not significant, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. Values are means +/- SEM.



Supplementary Figure 10. Determination of CEACAM5 gene promoter activity upon *Hp* infection.

DNA fragments of 0.4 kb (**a**) or 3.1 kb (**b**) upstream of the CEACAM5 gene containing the CEACAM5 promoter region were cloned in the vector pGL4.21[Luc2P/Puro] (Promega). AGS cells were co-transfected with the construct pGL4.21-CEACAM5 promoter 0.4 kb (**a**) or pGL4.21-CEACAM5 promoter 3.1 kb (**b**), and the internal control pGL4.74[hRLuc/TK] at a ratio of 1:1. Transiently transfected AGS cells were infected with *Hp* P12 wt or isogenic mutant strains (MOI 60, 37ºC, 5% CO_2, 3 h), or left without infection as the transfection controls. Promoter-less controls were AGS cells co-transfected with empty vector pGL4.21 and internal control pGL4.74 at a ratio of 1:1 or left without infection. After cell lysis, luciferase activities (firefly luminescence and renilla luminescence) were measured with the Dual-Glo Luciferase Assay System (Promega). Transfection control was normalized as 1. Values shown are derived from 3 (**a**) or 5 (**b**) independent experiments and are presented as mean values with standard deviation. One-way Anova test was used for statistic calculations.

Supplementary Table 1: Bacterial strains used in this study:

Strain	Plasmid	CEACAM binding protein	Reference
Moraxella strains			
Moraxella catarrhalis str. ATCC 43617		UspA1 negative	American Type Culture Collection
Moraxella catarrhalis str. ATCC 25238		UspA1 positive	American Type Culture Collection
Hp strains and mutants			
P12	pHel4	HopQI	[41]
P12-GFP	pCH7	HopQI	this work
P12Δ*hopQ*-GFP	pVK13, pCH7	--	this work
P12Δ*hopQ*	pVK13	--	this work
P12Δ*hopQ*::*hopQI*	pCE39	HopQI	this work
P12Δ*babA*	pLH3	HopQI	this work
P12Δ*babB1*Δ*babB2*	pLH5, pLH7	HopQI	this work
P12Δ*sabB1*Δ*sabB2*	pVK5, pVK6	HopQI	this work
P12Δ*hopZ*	pVK1	HopQI	this work
P12Δ*cagI*		HopQI	[6]
B8		HopQI	[42]
26695		HopQI	[43]
26695Δ*hopQ*		-	[44]
X47		HopQII	[45]
SS1		HopQII	[46]
PMSS1		HopQI / HopQII	this work
J99		HopQI	[47]
P322		HopQI	this work
P322Δ*hopQ*	pVK13	-	this work

Supplementary Table 2: Primer sequences used for cloning:

Gene	Primer	Sequence (5'→3')	Product
babA	LH1	GATCGGATCCGCTTGATATCGAATTCCTG	upstream
	LH2	GATCGGATCCATCCACGTTGAAAATCTC	
	LH3	GATCCTCGAGGTAGTTGGTTTAAGCGGTTG	downstream
	LH4	GATCATCGATCTTAAATCCCTTTGTGGAAC	
babB1	LH7	GATCCTCGAGATGCCGGCATTAGTAAAAAG	upstream
	LH8	GATCATCGATCTTAAATCCCTTTGTGGAAC	
	LH9	GATCCGCGGCCGCGAGAGAGTAAAAGGGTTTTC	downstream
	LH10	GATCCCGCGGCAAACGCTATGCAAGATGG	
babB2	VK23	GATCCTCGAGTTAACGGGCTTAAGAATTGG	upstream
	VK24	GATCATCGATCTTAAATCCCTTTGTGGAAC	
	VK25	GATCGCGGCCGCCCTTTTTGGATTAAATTAGG	downstream
	VK26	GATCCCGCGGTTGAAACTAAGGAGAATGC	
sabB1	VK9	GATCCTCGAGTTTAGACAACAAGGAATTGG	upstream
	VK10	GATCATCGATTTGTAAACATGAATAGAAATG	
	VK11	GATCGCGGCCGCGCTTCATCAAATCTATTTTG	downstream
	VK12	GATCCCGCGGAAGGTGGTCCATAATGAG	
sabB2	VK13	GATCCTCGAGTATAGTATAGTAAAACATCG	upstream
	VK14	GATCATCGATGAGTGATTCAAGCTCTC	
	VK15	GATCGCGGCCGCTAACTATTTATCTTTAGAGTG	downstream
	VK16	GATCCCGCGGCAAGAAATGTTGCAAAGTG	
hopZ	VK1	GATCCTCGAGCCAGGCTTTTAGATAATTTG	upstream
	VK2	GATCATCGATGTTAAAACCCTTTGTGAAAC	
	VK3	GATCGCGGCCGCCTTTGAAATTAAATTGAGTG	downstream
	VK4	GATCCCGCGGGGCTTGAGTCTAGAAAAC	
hopQ	VK57	GATAGTCGACAGGCGAGAATGGAAGTGATTAAG	upstream
	VK58	TGATTGAACGCGGATCCTTTAAGGTATAATGTTT CCGCCAC	
	VK59	TATACCTTAAAGGATCCGCGTTCAATCAAGCTTG TAAGAG	downstream
	VK60	GATCGAATTCTCCTTTAAGGGTGAGCTACATTG	
CEACAM5	macCEACAM5-IF-sense	GAAGTTATCAGTCGACACCATGGGGTCTCCCTCAGCC	upstream
	CEACAM5-NT-IF-antisense	ATGGTCTAGAAAGCTTTGCTTGAAGTACTCTGGGTGT ACATGGAACTGTCCGGTTGC	downstream
CEACAM5-IgC	LH90	GCATCTGGAACTTCTCCTGGTCTCTC	IgC deletion of CEACAM5
	LH91	GTATACCCGGAACTGGCCAG	
CEACAM5-IgV	LH79	TTCCGGGTATACCCGGAG	IgV deletion of CEACAM5
	LH89	GAGCTTGGCAGTGGTGG	
MBP-CEACAM5	pDAB182FP	GCGCGCCCATGGCCAAGCTCACTATTGAATCCA CGCCG	MBP-CEACAM5 fusion protein
	pDAB182RP	ATATATCTCGAGTTACAGTTCCGGGTATACCCGG AACTGGCCAGTTGCTTC	
MBP-*hopQ*	pDAB203FP	ATATATGCGGCCGCGGGCGTTTTTTTAAGCGTG GGTTATCAAATTG	MBP-HopQ fusion protein
	pDAB203RP	GCGCGCCTCGAGCGTGGTTGAAGAAGCGATCAT GCCC	

Supplementary Table 3: Thermodynamic parameters of binding between MBP-HopQ and CEACAM variants

	N	Kd (nM)	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	TΔS (kcal mol^{-1})
CEACAM1	0.59±0.01	23±1	-19.8±0.1	-31.5±0.1	-9.4
CEACAM3	0.63±0.00	268±4	-23.0±0.6	-47.1±2.1	-14.0
CEACAM5	0.63±0.01	61±3	-30.5±0.3	-69.4±1.2	-20.7
CEACAM8			No binding		

All values are means of duplicate runs

Supplementary Table 4:
Plasmids used for transfection of eukaryotic cells or construction of Hp mutant strains:

Plasmid	Gene product	Reference
pRc/CMV-CEA	CEACAM5	[37]
pLH45	CEACAM5ΔIgC	this work
pLH46	CEACAM5ΔIgV	this work
pRc/CMV-CEACAM1	CEACAM1	W. Zimmermann, unpublished
pRc/CMV-CEACAM4	CEACAM4	[48]
pRc/CMV-CEACAM6	CEACAM6	W. Zimmermann, unpublished
pRc/CMV-CEACAM8	CEACAM8	W. Zimmermann, unpublished
pCDNA3-CEACAM3	CEACAM3-HA	[33]
CEA-N-GFP plasmids		[36, 24]
pVK1	deletion plasmid *hopZ*	this work
pVK5	deletion plasmid *sabB1*	this work
pVK6	deletion plasmid *sabB2*	this work
pVK13	deletion plasmid *hopQ*	this work
pLH3	deletion plasmid *babA*	this work
pLH5	deletion plasmid *babB1*	this work
pLH7	deletion plasmid *babB2*	this work
pCE39	genetic complementation *hopQ* (P12)	[18]

Fig. 1b, Start extract

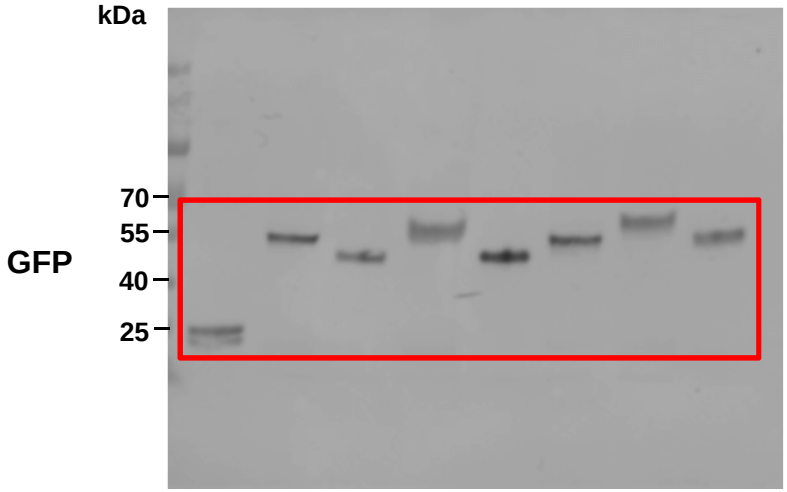


Fig. 1b, RecA

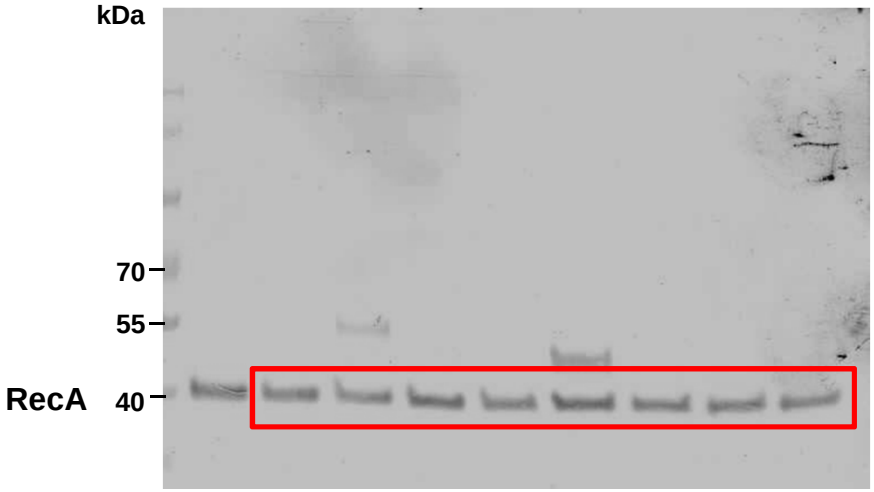


Fig. 1b, Pulldown

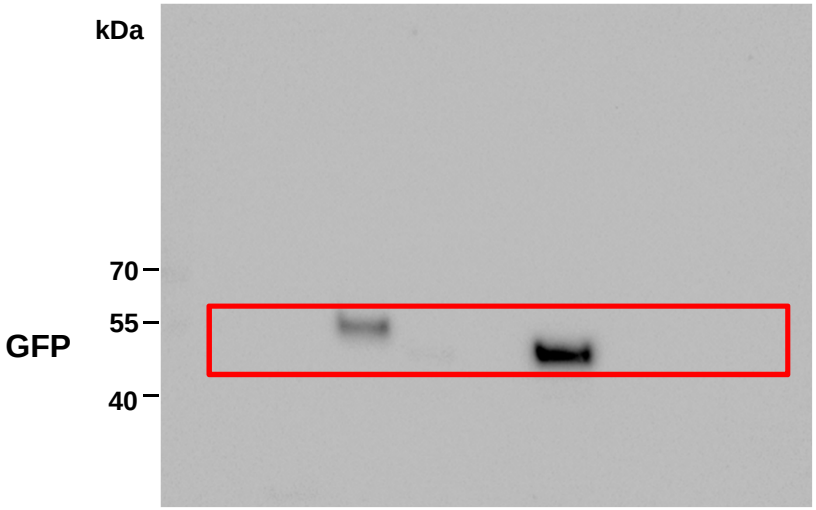


Fig. 2b, left part

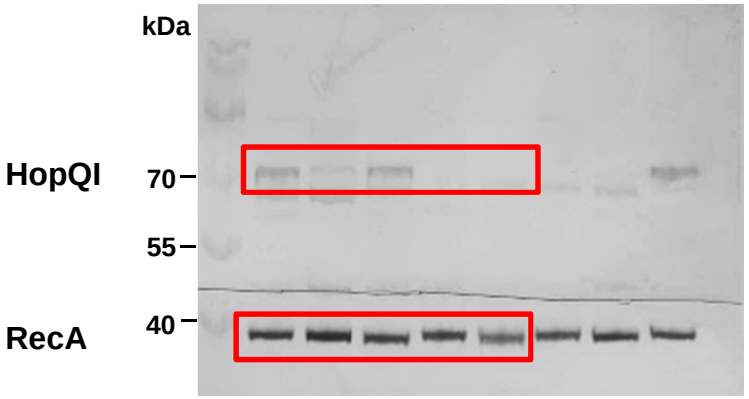


Fig. 2b, right part

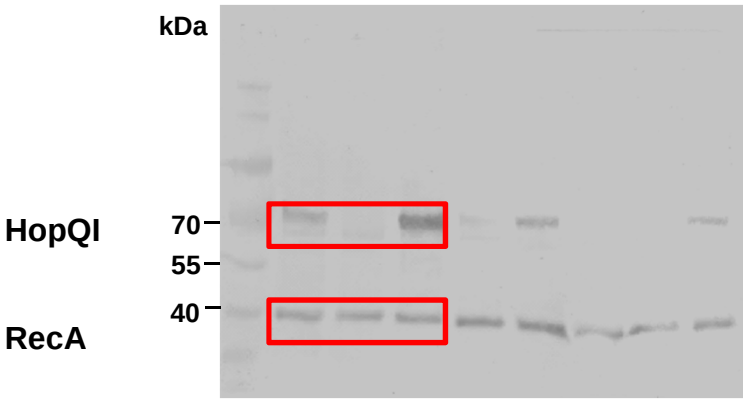


Fig. 3a

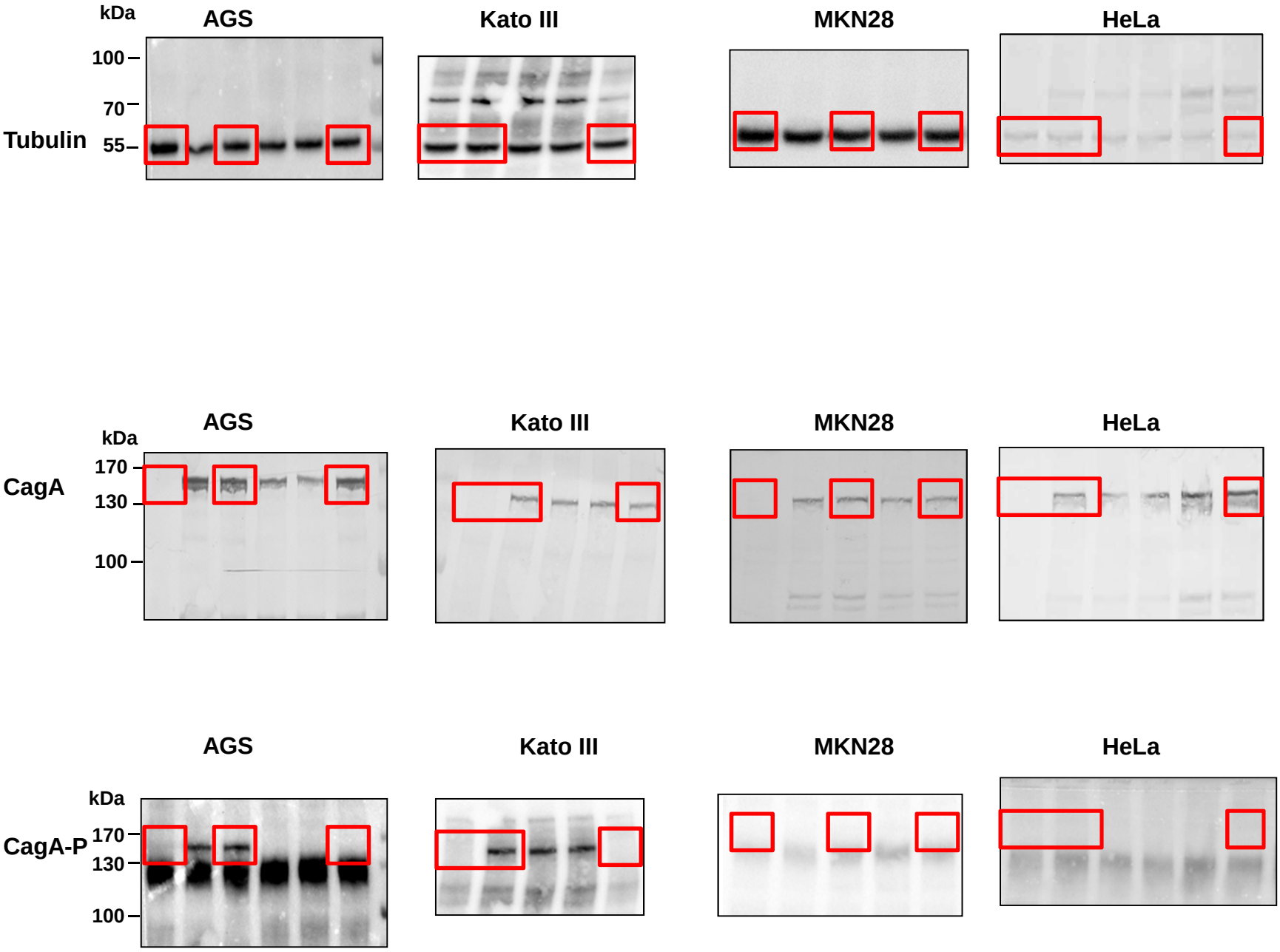

kDa
AGS
Kato III
MKN28
HeLa
100
70
55
Tubulin
AGS
kDa
Kato III
MKN28
HeLa
170
130
100
CagA
AGS
Kato III
MKN28
HeLa
kDa
170
130
100
CagA-P

Fig. 3a

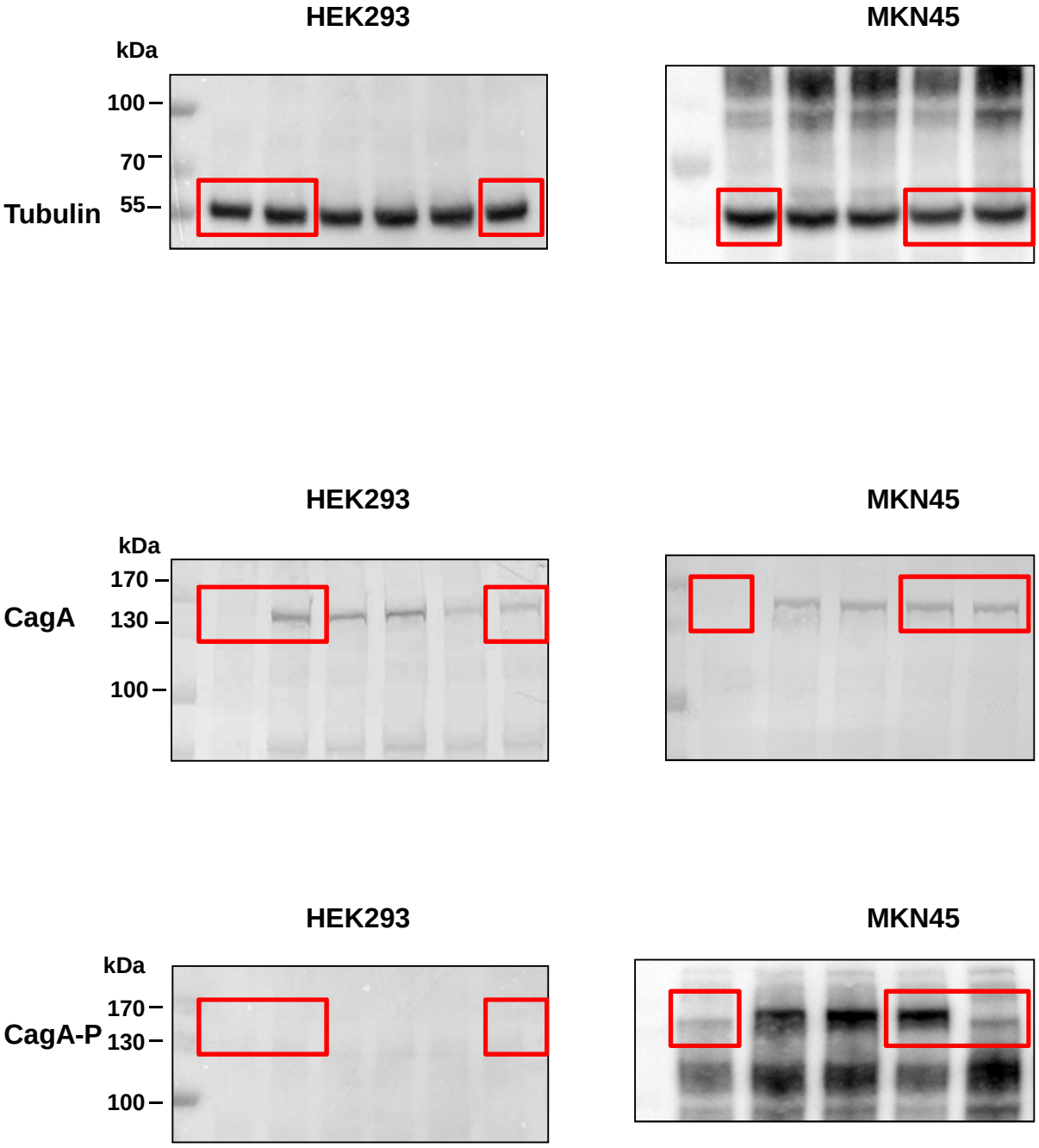

HEK293
MKN45
kDa
100
70
55
Tubulin
HEK293
MKN45
kDa
170
130
100
CagA
HEK293
MKN45
kDa
170
130
100
CagA-P

Fig. 3b

Fig. 3c

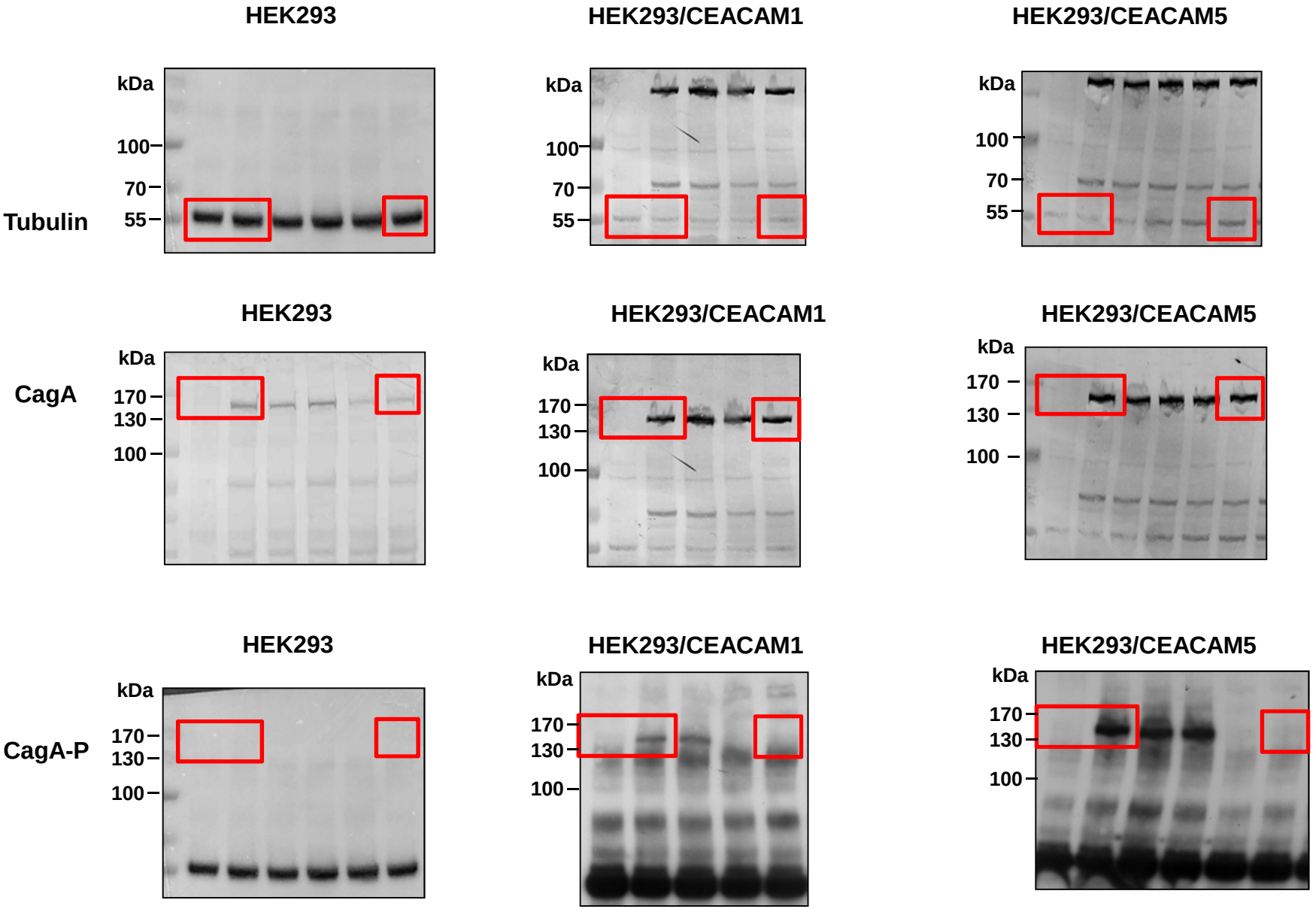

HEK293
HEK293/CEACAM1
HEK293/CEACAM5
Tubulin
CagA
CagA-P
kDa
100
70
55
170
130

Fig. 4c

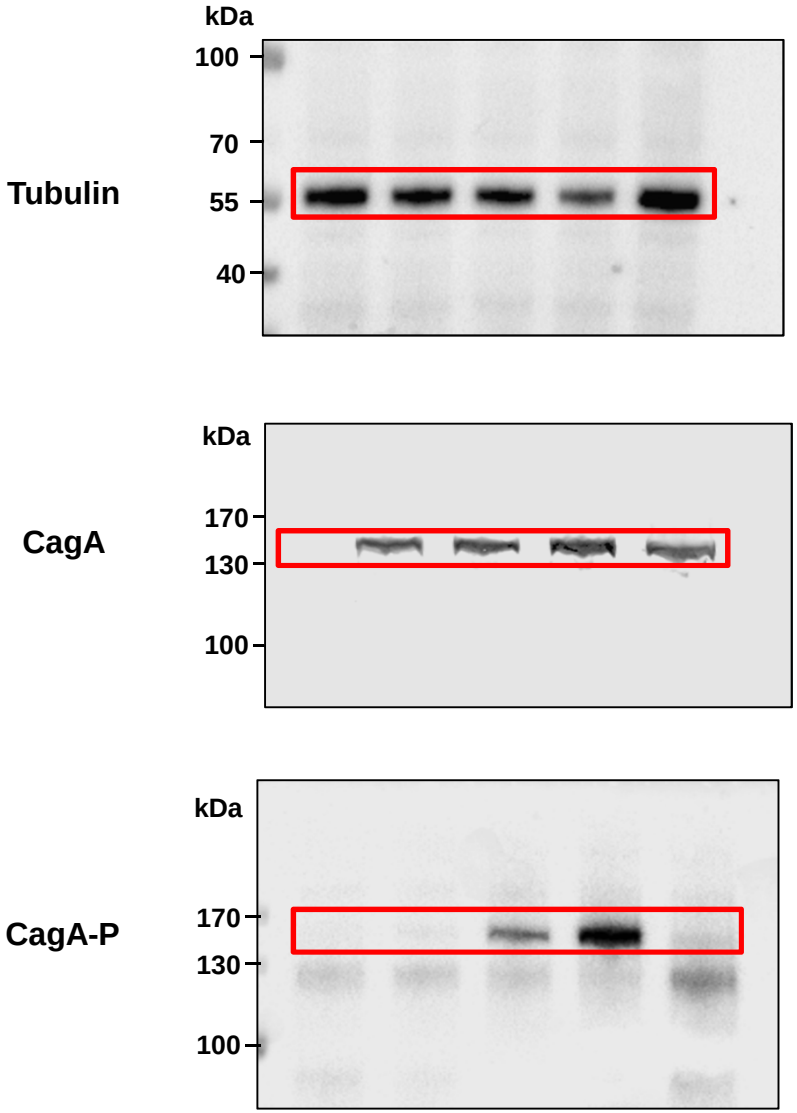

kDa
100
70
55
40
Tubulin
kDa
170
130
100
CagA
kDa
170
130
100
CagA-P

Fig. 4e

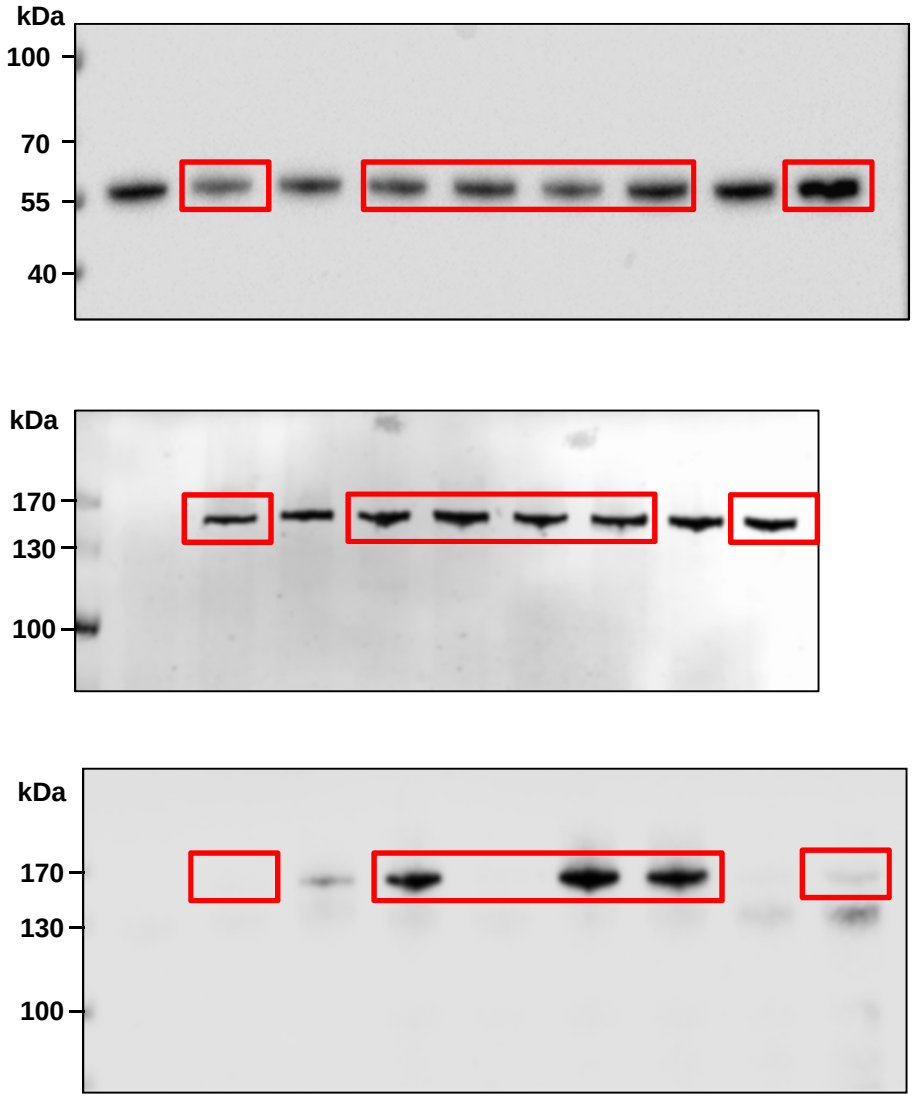

kDa
100
70
55
40
kDa
170
130
100
kDa
170
130
100

Fig. S4a

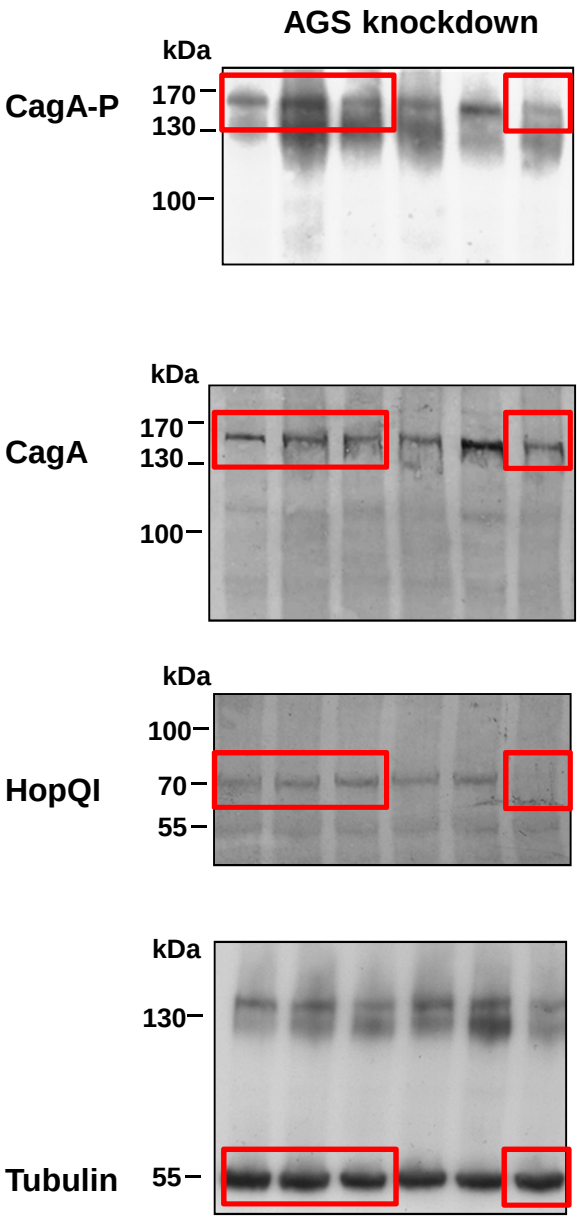


Fig. S4b

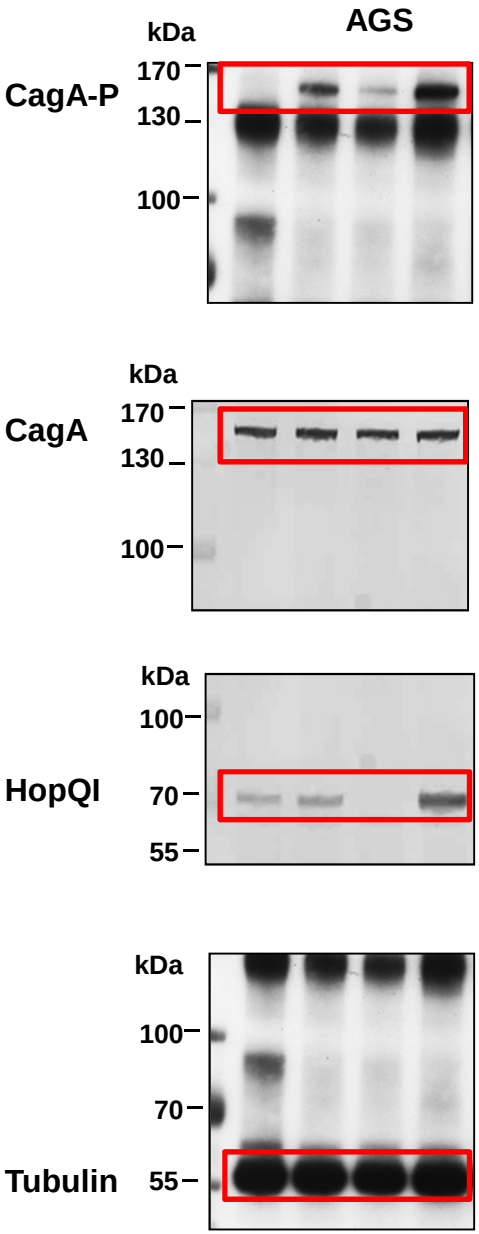
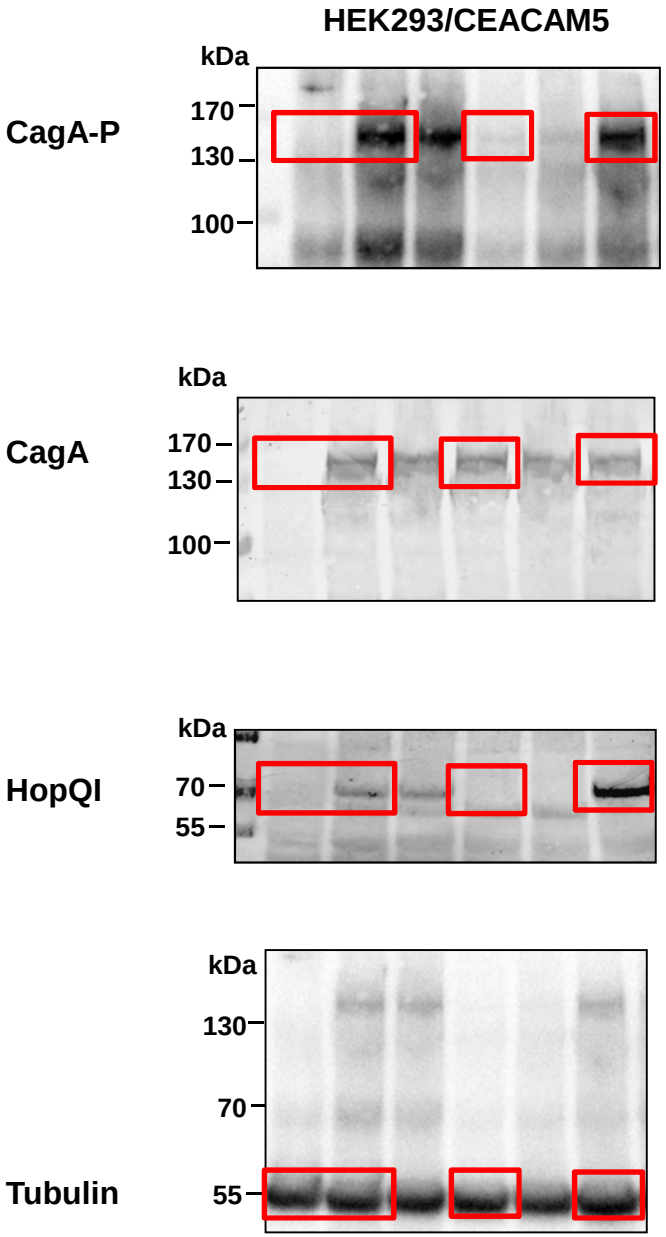
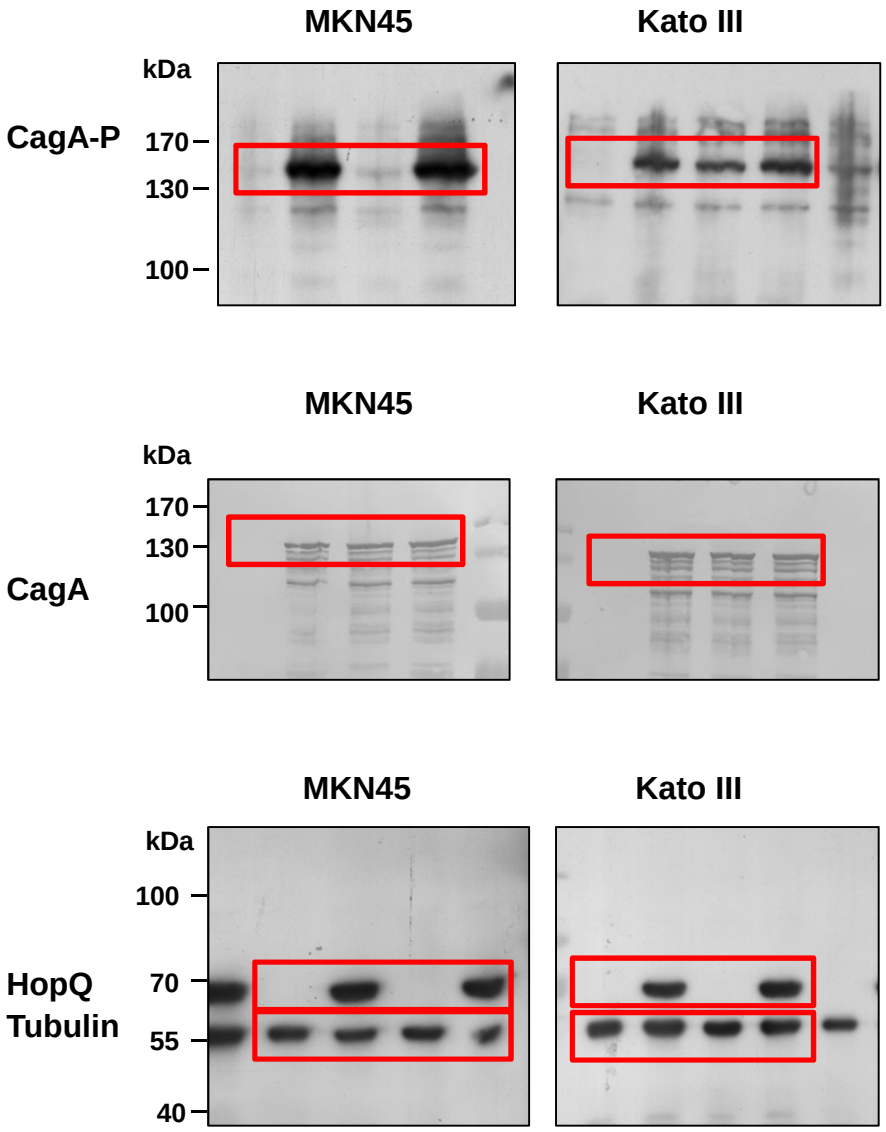


Fig. S4c




MKN45
Kato III
kDa
CagA-P
170
130
100
MKN45
Kato III
kDa
CagA
170
130
100
MKN45
Kato III
kDa
HopQ
Tubulin
100
70
55
40