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Short Communication

Thioloxidoreductase HP0231 of *Helicobacter pylori* impacts HopQ-dependent CagA translocation

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ABSTRACT

Thioloxidoreductase HP0231 of *Helicobacter pylori* plays essential roles in gastric colonization and related gastric pathology. Comparative proteomics and analysis of complexes between HP0231 and its protein substrates suggested that several Hop proteins are its targets. HP0231 is a dimeric oxidoreductase that functions in an oxidizing Dsb (disulfide bonds) pathway of *H. pylori*. *H. pylori* HopQ possesses six cysteine residues, which generate three consecutive disulfide bridges. Comparison of the redox state of HopQ in wild-type cells to that in *hp0231*-mutated cells clearly indicated that HopQ is a substrate of HP0231. HopQ binds CEACAM1, 3, 5 and 6 (carcinoembryonic antigen-related cell adhesion molecules). This interaction enables T4SS-mediated translocation of CagA into host cells and induces host signaling. Site directed mutagenesis of HopQ (changing cysteine residues into serine) and analysis of the functioning of HopQ variants showed that HP0231 influences the delivery of CagA into host cells, in part through its impact on HopQ redox state. Introduction of a C382S mutation into HopQ significantly affects its reaction with CEACAM receptors, which disturbs T4SS functioning and CagA delivery. An additional effect of HP0231 on other adhesins and their redox state, resulting in their functional impairment, cannot be excluded.

1. Introduction

The bacterial proteins of the Dsb (*disulfide bonds*) family — important components of the posttranslational protein modification system — catalyze the formation of disulfide bridges, a process that is crucial for protein structure stabilization and protein activity. Dsb proteins play an essential role in the assembly of many virulence factors. The Dsb system of *Helicobacter pylori* is less complicated than that described in detail for *Escherichia coli*. The *H. pylori* genome lacks classical DsbA and DsbB proteins, which are members of the Dsb oxidative pathway, as well as classical DsbC and DsbD proteins that are members of the Dsb isomerization pathway. It includes only two periplasmic proteins (HP0231 and HP0377) and a membrane located DsbB-like protein, HP0595 (Bocian-Ostrzycka et al., 2015a, b).

The dimeric oxidoreductase (HP0231), a V-shaped protein, functions in an oxidizing pathway of *H. pylori* (Yoon et al., 2011; Roszczenko et al., 2012; Lester et al., 2015). In order to introduce disulfide bonds, most DsbA proteins need to cooperate with a membrane DsbB that transfers electrons to the respiratory chain, providing the re-oxidation of the DsbA. It is still unclear how HP0231 is re-oxidized *in vivo*. *H. pylori* does not encode a classical DsbB, though it encodes a DsbB-like protein, HpDsbI (HP0595), which lacks the second pair of Cys residues located in the 3–4 periplasmic loop of classical DsbBs that are involved in cooperating with DsbA. Although mutation of the *hpdsbI* gene has an impact on the redox states of HP0231 and HP0377, it does not influence HP0231 function in motility (Roszczenko et al., 2012, 2015). Resolved structure and biochemical analysis of HP0377 has documented that it is a counterpart of CcmG (*cytochrome c maturation*) (Yoon et al., 2013). HP0377, in contrast to other CcmGs described so far, is a bifunctional protein, acting not only in the

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reduction of apocytochrome c but, additionally, in disulfide isomerization (Roszczenko et al., 2015).

The Dsb system plays an important role in *H. pylori* pathogenesis. We demonstrated that an *H. pylori* mutant impaired in disulfide bond formation (*hp0595*-mutated strain) is greatly impaired in its ability to colonize the mouse gastric mucosa (Godlewska et al., 2006). Recently, work conducted by Zhong et al. showed that the lack of *H. pylori* HP0231 impairs CagA translocation into gastric epithelial cells, reduces VacA-induced cellular vacuolation and influences the level of *H. pylori*-induced IL8 induction in epithelial cells. Moreover, *H. pylori* HP0231-deficient bacteria were not able to colonize the gastric mucosa of mice (Zhong et al., 2016).

Recently, it was shown that not only periplasmic, but also outer membrane proteins, are substrates of the Dsb system (Denoncin et al., 2010). The *H. pylori* genome encodes numerous outer membrane proteins (OMPs). Most OMPs can be allocated to two protein families: *Helicobacter* OMPs (Hop) and Hop-related proteins (Hor). One of the Hops is HopQ (Omp27), which possesses six cysteine residues that form three disulfide bonds (Javaheri et al., 2016). HopQ binds CEACAM1, 3, 5 and 6 and induces host cell signaling. This interaction enables translocation of CagA into host cells (Koniger et al., 2016). HopQ is also involved in the inhibition of leukocyte migration in a CagA-dependent process (Busch et al., 2015). We asked the question: does HP0231 affect CagA translocation through an impact on HopQ posttranslational modification.

2. Materials and methods

2.1. Bacterial strains, primers, plasmids, media and growth conditions

Bacterial strains, plasmids and primers used in this study arelisted in Tables S1 and S2.

2.2. DNA/RNA techniques

2.2.1. General DNA manipulations

Standard DNA manipulations were carried out as described earlier (Sambrook and Russell, 2001) or according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed with PrimeStar HS DNA Polymerase (Takara), HotStarHiFidelity Polymerase (Qiagen) or Phusion (Thermo Scientific) under standard conditions. Synthetic oligonucleotides synthesis and DNA sequencing were performed by Genomed S.A., Warsaw, Poland.

2.2.2. Site-directed mutagenesis of the hopQ, hp0377 and hp0231 genes

To obtain mutated HopQ proteins, a set of recombinant plasmids was constructed from pCE4, which carries the *hopQ* gene. Cys-to-Ser point mutations were generated using the Quick Change Site-Directed Mutagenesis Kit (Qiagen) according to the manufacturer's instructions, starting with 100 ng of pCE4 template and 125 ng of each primer (listed in Table S2). To obtain C92S-mutated HP0377 protein, a recombinant plasmid was constructed from pUWM2065 carrying the *hp0377* gene with its promoter, signal sequence and a His-tag at the C-terminus. To obtain P258T mutated HP0231, the recombinant plasmid pUWM589 was used as a template. The Cys-to-Ser mutation of HP0377 and the Pro-to-Thr were generated as described above. Appropriate primer pairs for site-directed mutations are listed in Table S2. In all cases the correct construction of recombinant plasmids was verified by sequencing.

2.2.3. Natural transformation of H. pylori

The naturally competent *H. pylori* P12 was grown on horse serum agar plates for 24 h. Subsequently, bacteria were plated onto fresh plates for 5 h. Then 0.5–1 μg of plasmid DNA was added and plates were incubated for 24 h. Afterwards, bacteria were transferred onto a plate supplemented with kanamycin or chloramphenicol/kanamycin, and transformants were grown for 4–5 days.

2.2.4. Real-time qPCR

RNA was isolated using the GeneJET RNA Purification Kit (ThermoScientific), according to the manufacturer's protocol. Real-time qPCR was performed using the Power SYBR Green RNA-to-CT 1-Step Kit (Life Technologies), on an ABI Prism 7900 H sequence detection system (Applied Biosystems). Reactions were performed in 25 μl containing 20 ng RNA, 10 μl SYBR Green mix, 0.16 μl RT mix and 0.2 μM per primer. Program: 30 min 48 °C; 10 min 95 °C; followed by 40 × cycles of 15 s 95 °C/60 s 60 °C. For each oligonucleotide pair and RNA sample, the reaction was performed in triplicate. The amplification plots obtained from the RT–PCR were analyzed with the ABI Prism SDS Software package (version 2.2.2; Applied Biosystems). The expression levels were quantified applying the comparative C_t (threshold cycle) method and calculating $\Delta\Delta C_t$. Relative expression levels of the target genes were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase in each individual sample. Fold change was calculated as an average of $\Delta\Delta C_t$ of independent biological replicates ($2^{-}\Delta\Delta C_t$), and the standard deviation (SD) was calculated as $\Delta\Delta C_t + s$ and $\Delta\Delta C_t - s$, where s is the pooled SD of the ΔC_t and ΔC_t control values.

2.3. Protein analysis

Preparation of *H. pylori* and *E. coli* protein extracts, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and blotting procedures were performed by standard techniques (Sambrook and Russell, 2001). For Western-blot analysis we used rabbit specific anti-HP0231 (Roszczenko et al., 2012), rabbit specific anti-HP0595 (Roszczenko et al., 2015), rabbit specific anti-HP0377 (Roszczenko et al., 2015) and a peptide-based serum (AK298) directed against the HopQ type I- specific protein region (Koniger et al., 2016).

2.3.1. Preparation of subcellular fractions

The localization of HopQ and HP0377 proteins was determined as previously described (Roszczenko et al., 2015).

2.3.2. Overexpression and purification of HP0231 (its C137S and P258T variants)

The expression vector pUWM2191, carrying HP0231-P258T-His6 (Table S1), was constructed by cloning the insert from pUWM2190 into pET28a, using NcoI and XhoI restriction enzymes. For further experiments, proteins were overexpressed by auto induction from an *E. coli* Rosetta strains and purified using the NGC Medium-Pressure Chromatography Systems, Bio-Rad, as previously described (Roszczenko et al., 2015).

2.3.3. Identification of HP0231 protein substrates

The mutated forms of $HP0231_{P258T}$ and $HP0231_{C137S}$ (KBO2068 strain) generated by site-directed mutagenesis were overexpressed in an*E. coli* Rosetta strain. Substrate-HP0231 complexes were purified using NGC™ Medium-Pressure Chromatography. First, His-tagged proteins were immobilized on an Ni-NTA column. Then, lysate of *H. pylori* 26695 was loaded on to the column. Complexes were eluted from the column with increasing concentrations of imidazole and analyzed by SDS-PAGE under reducing and non-reducing conditions.

2.3.4. In vivo redox state of HopQ in H. pylori wt and its hp0231 mutated version

The redox states of HopQ was visualized by alkylating the free cysteine residues using 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS, Invitrogen) as previously described (Denoncin et al., 2013; Roszczenko et al., 2015). The proteins were resolved by 12% SDS-PAGE without reducing agent. HopQ was then detected by an immunoblot analysis using peptide-based anti-HopQ serum. As controls, we used samples treated with DTT before precipitation of the proteins with TCA.

2.4. Analysis of the T4SS functioning - CagA delivery into host cells

2.4.1. Infection assays

For infection experiments, AGS cells were grown in 12-well tissue culture plates for 1–2 days to reach 70 to 80% confluence. AGS cells were then incubated in serum-free RPMI-1640 medium (Gibco) for 3 h before infection. Bacteria harvested from agar plates were washed two times in PBS, resuspended in RPMI medium and added to AGS cells at an assay–specific multiplicity of infection (MOI). Infection was carried out at 37 °C in a humidified atmosphere supplemented with 5% CO_2. Samples were taken 3 h and 6 h post infection (p.i).

2.4.2. Hummingbird phenotype

Development of the hummingbird phenotype in AGS cells (human gastric adenocarcinoma cell line, ATCC CRL-1739) was assessed upon infection with *H. pylori* strains at an MOI of 100. Samples were analyzed after 6 h of infection. AGS cells were fixed in 2% paraformaldehyde, treated with 0.2% Triton X100 in PBS for 20 min at RT (room temperature) and incubated with Alexa Fluor® 546 (A546) phalloidin (Invitrogen, Germany) and DAPI to stain actin and DNA, respectively. Cells were visualized using a Leica TCS SP laser confocal microscope. Elongated cells were defined as cells that had thin needlelike protrusions that were > 20 µm long and a typical elongated shape. The proportion of elongated cells was calculated the numbers of cells having the hummingbird phenotype.

2.4.3. Immunoblotting

Cells were lysed in 1× Laemmli buffer (60 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue). Proteins were separated by SDS-polyacrylamide gel (12%) electrophoresis and then transferred onto PVDF membranes (Perkin Elmer) in buffer (25 mM Tris, 192 mM glycine, 20% methanol). Non-specific binding sites were blocked by incubation in blocking buffer (0.05 M Tris (pH 7.4), 0.2 M NaCl, 3% Tween, 3% BSA) for 1 h at RT before probing with primary antibodies against β-actin, (Sigma, Germany), phospho-Tyr (PY99) and CagA, Santa Cruz Biotechnology, Germany); all at a final dilution of 1:1000 in blocking buffer supplemented with 3% BSA. Secondary HRP-labelled goat anti-rabbit or goat anti-mouse antibodies (GE Healthcare, Germany) were used at a dilution of 1:3000 in the same buffer. The blots were detected on the X-ray film AmershamHyperfilm™ ECL (GE Healthcare) after treatment with enhanced chemiluminescence reagent (ECL) (Pierce, USA).

2.5. Bacterial pull down

Analysis of the HopQ and its mutated variants interactions with human His-tagged CEACAM1 (10822-H08H, Sino Biological) by pull-down experiment was performed as described (Javaheri et al., 2016).

2.6. M/S analysis

The quantitative, label-free proteomic analysis of periplasmic fractions from different *H. pylori* strains (wild type and mutant strain *hp0231*) and analysis of complexes between HP0231 and its protein substrates were performed at the Mass Spectrometry Laboratory Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). The details of experiments are given in supplementary materials.

The given annotations (*hp0231*, *hp0377*, *hp0595)* concern the *H. pylori* 26695 strain, and the gene numbers for the *H. pylori* P12 strain are *hpp12_0231*; *hpp12_1044* and *hpp12_0602*, respectively.

3. Results and discussion

The influence of HP0231 on *H. pylori* virulence was documented by Zhong (Zhong et al., 2016). We first investigated whether other components of the *H. pylori* Dsb network also have an impact on pathogen interaction with epithelial cells. Isogenic *hp0231* and *hp0595*(*dsbI*) knock-out strains of *H. pylori* P12 were constructed by allelic exchange methodology using previously prepared constructs (Raczko et al., 2005; Roszczenko et al., 2012).The absence of *hp0595* and *hp0231* gene products was confirmed by Western blot analysis (Fig. S1AB). As HP0377 is an essential protein for *H. pylori,* it is impossible to directly evaluate its potential influence on *H. pylori* virulence. To overcome this difficulty, we expressed *in trans,* in a wt *H. pylori,* a mutated version of His-tagged HP0377 containing the CSYC motif changed to CSYS. The rationale for using $HP0377_{CXXS}$ variant was based on the observation that thiol oxidoreductases of reductase activity with changed catalytic motif CXXC to CXXS create stable complexes with their targets. Thus, it leads to the reduction of the Dsb substrate physiological activities (Denoncin et al., 2010; Jameson-Lee et al., 2011). The presence of $HP0377_{CSYS}$ overexpressed from the plasmid was confirmed by Western blot analysis using mouse specific anti-His and rabbit specific anti-HP0377 sera (Fig. S1CD). The generated strains were used to estimate CagA translocation into gastric epithelial cells. *H. pylori* wild type bacteria, the *hp0595* mutant and $hp0377_{CXXS}{}^{+}$ induced a hummingbird phenotype. No such phenotypic changes were induced in cells infected with bacteria lacking *hp0231*, whereas complementation of *hp0231* reconstituted the characteristic cell elongation (Fig. 1). To further analyze CagA translocation by strains with the *hp0231* and *hp0595* deletions, or with $HP0377_{CXXS}$ overexpression, lysates from infected cells were subjected to Western blot to detect CagA phosphorylation. Infection of cells with wild type, Δ*hp0595* and $hp0377_{CXXS}{}^{+}$ bacteria resulted in CagA tyrosine phosphorylation, while in the absence of *hp0231*, phosphorylation of CagA was not detected (Fig. 2). Also, only the *H. pylori* P12 strain lacking HP0231 was deficient in IL-8 induction in AGS cells upon infection, as measured by Real time qPCR (Fig. 3). These data, consistent with those presented by Zhong et al., suggested that CagA translocation is impaired only in the absence of HP0231 (Zhong et al., 2016).

Our comparative analysis of the periplasmic subproteome of *hp0231* mutant indicated two main differences between wt and the HP0231 deficient strain (Table 1). First, the levels of many oxidative stress combating enzymes, such as catalase or superoxidase dismutase, were lower in the proteome of the *hp0231* mutant compared to the wt strain. This may suggest a role of the Dsb system in defense against oxidative

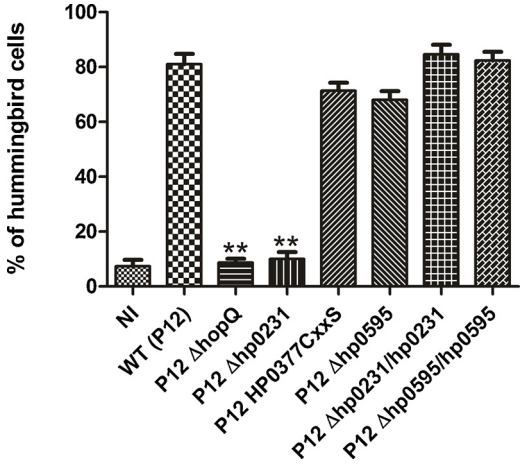

Fig. 1. Development of hummingbird phenotype in AGS cells upon infection with the *H. pylori* wild type strain P12 (WT) and with its isogenic mutants P12Δ*hopQ*, P12Δ*hp0231*, P12Δ*hp0595*, P12*hp0377*$_{CXXS+}$ and the complemented mutants: P12Δ*hp0231/hp0231*, P12Δ*hp0595/hp0595*,in comparison to non-infected AGS cells (NI). The results are the means of three independent experiments. The error bars indicate standard deviations. Statistical analysis was evaluated using Student's t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with WT P12 infected cells.

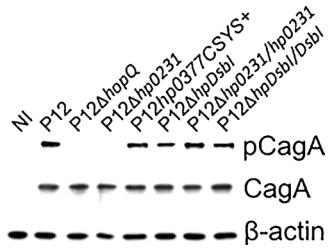

Fig. 2. CagA phosphorylation in AGS cells upon infection with H. pylori wild type strain P12 (WT), with its isogenic mutants P12Δ*hopQ*, P12Δ*hp0231*, P12Δ*hp0595*, P12*hp0377*$_{CXXS}$$^{+}$and the complemented mutants: P12Δ*hp0231/hp0231*, P12Δ*hp0595/hp0595* in comparison with non-infected AGS cells (NI). Infection was performed at an MOI of 100 for 3 h. Phosphorylated CagA (pCagA), total amount of CagA (CagA) and AGS cells (β-actin) were estimated with specific antibodies by immunoblot analysis.

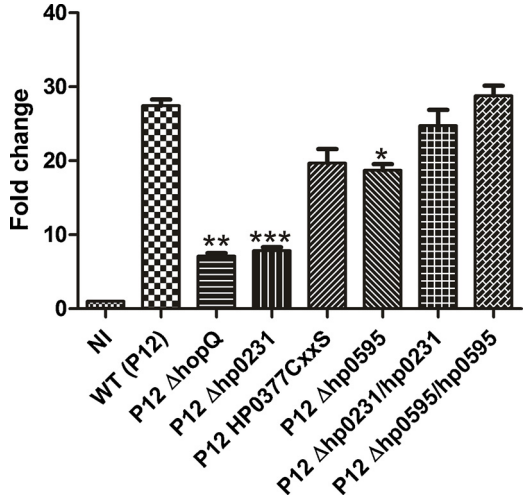

Fig. 3. Production of IL-8 in AGS cells upon infection with the *H. pylori* wild type strain P12 (WT), its isogenic mutants: P12 Δ*hop*, P12 Δ*hp0231*, P12 *hp*0377$_{cxxs+}$,P12Δ*hp0595*, and P12 Δ*hp0231/hp0231*, P12 Δ*hp0595/hp0595* in comparison to non-infected AGS cells (NI) estimated by RT-PCR. Errors bars show ± SD of three independent experiments. Statistical analysis was evaluated using Student's t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with WT P12 infected cells.

stress. It should be pointed out that the localization of these proteins is still unclear. According to *in silico* predictions, they should be localized in the cytoplasm. However, many proteomic analyses have documented their extracytoplasmic location (Sabarth et al., 2002; Backert et al., 2005; Smith et al., 2007). Second, we noticed that there was a significant increase in the amount of HopQ and HopD present in the periplasmic space of an *H. pylori hp0231*-mutated strain, as compared to the wt strain. This observation may indicate that HopQ/HopD lacking disulfide bonds cannot be transported into cell's outer-membrane. To substantiate this finding, a subcelullar fractionation experiment was carried out for *H. pylori* wt and *hp0231*-mutated strains in order to determine the location of HopQ. First we asked the question whether the periplasmic protein fraction was not contaminated by the membrane proteins. As an extra control for the cellular fractionation methodology, we traced the cellular location of HP0377, which is an *H. pylori* inner-membrane lipoprotein (Roszczenko et al., 2015). Localization of HopQ and HP0377 within specific subcellular fractions was determined by immunoblot analysis using specific anti-HopQ and anti-HP0377 sera, respectively. We found that in the wt cell fractionation experiment HopQ (outer membrane, surface-exposed) and HP0377 (inner-membrane) were not present among periplasmic proteins. Thus, we became convinced that the used procedure was correct. As mentioned above, we observed that HopQ, in agreement with expectations, was present only in the outer-membrane compartment of the wt strain. In contrast, in the *hp0231*-mutated strain most of the HopQ was detected in the periplasmic compartment (Fig. 4A). HP0377 was present in the inner membrane fraction of the wt as well as of all analyzed mutated strains (Fig. S2). Thus, we conclude that the observed presence of HopQ in the periplasmic compartment of the mutated strains probably results from the lack of disulfide bonds. Additionally, the observed phenotypic differences of AGS cells infected by *H. pylori* lacking HP0231 resemble those that arise from infection with a strain lacking *hopQ:* both phenotypes abolish CagA delivery, IL-8 release and cell elongation.

Next, to confirm the interaction between Hop proteins and HP0231, we identified complexes formed between HP0231 and its potential substrate proteins using site-directed mutagenesis. The mixed complexes between thioloxidoreductases and their substrates are short-lived and are difficult to detect, and the *cis*-Pro loop is highly conserved in thioloxidoreductases. It has been documented that mutations that alter the conserved *cis*-proline of thioloxidoreductases involved in disulfide bond generation, slows the second step of oxidative folding, which results in accumulation of their intermediate complexes with substrates (Kadokura et al., 2004; Jameson-Lee et al., 2011). Thus, to stabilize the intermediate complexes of HP0231 with its substrate proteins, we constructed a mutated version of HP0231 with proline 258 replaced by threonine. His-tagged HP0231 was overexpressed and purified from an *E. coli* Rosetta strain and used to identify complexes between HP0231 and its substrates by a "reverse purification" substrate trapping technique. A small scale experiment was first performed to verify the effectiveness of the method. Potential complexes, between *H. pylori* 26695 HP0231$_{P258T}$ and its protein substrates, eluted from the Ni-NTA affinity column with increasing concentrations of imidazole were subjected to SDS-PAGE with or without treatment by a reducing agent (DTT) and were next analyzed by immunoblot using anti-HP0231 antibodies (Fig. 5A). HP0231 was the most abundant protein present, but other proteins of higher molecular weight that reacted with anti-HP0231 serum were also observed. After DTT treatment, several of the additional protein bands present under the non-reducing conditions disappeared. So, we concluded that they correspond to complexes between HP0231 and its potential substrates. Next, in a large scale experiment, protein bands visualized by staining with Coomassie Blue (Fig. 5B) were cut out of a non-reducing gel, digested with trypsin and analyzed by mass spectrometry (details of the analysis are given in the methods section of the supplementary materials). In the control experiment another mutated version of HP0231$_{CXXS}$ was immobilized on an Ni-NTA column. As mentioned above Dsb oxidoreductases of reductase activity with CXXS motif creates complexes with their substrates. As HP0231 acts as oxidase, HP0231 variant possessing C-terminal cysteine of the CXXC motif changed to serine does not form complexes with HP0231 substrates. We compared proteins identified by MS in both experiments. Those identified in control experiment (HP0231$_{CXXS}$ immobilized in the column) with score higher than 50% of value observed when HP0231$_{P/T}$ was used as a bait were not considered as HP0231 substrates (Tables S3 and 2). Finally, MS analysis revealed several potential HP0231 substrate proteins, including seven Hop proteins containing well-conserved paired-cysteine patterns (Moonens et al., 2016). Two of them (HopS, HopU) potentially contain four disulfide bonds, three (HopT, HopQ, HopD) have three disulfide bonds and two (HopM, HopN) potentially contain two disulfide bonds (Table 2). According to data presented by Voss et al., all of them meet two or three criteria to be classified as surface exposed, outer membrane proteins (Voss et al., 2014). HopZ, which also contains conserved cysteine pairs and is a surface exposed protein, was not detected by our analysis, due to the phase variation phenomenon: the *H. pylori* 26695 *hopZ* gene contains 11 CT dinucleotides located in the region encoding the signal sequence, which results in a frameshift mutation. As a consequence, HopZ is not produced (Peck et al., 1999). Our analysis also did not indicate SabA (HopP; OMP16), which is essential for *H. pylori*

Table 1
Quantitative MS analysis comparing the periplasmic proteomes of *hp0231*::*cat* and wild type *H. pylori* 26695 strains.

locus	%WT (q-value)	Number of peptides	Number of Cysteines	localization	Protein name & function
HP0224	80 (0,00005)	34	4	cytoplasm	Peptide methionine sulfoxide reductase MsrA (oxidative stress response)
HP0073	117 (0,00005)	32	1	cytoplasm/secreted	UreA, Urease subunit alpha (catalysis of urea degradation to ammonia and carbon dioxide)
HP1286	80 (0,00005)	18	0	periplasm	Hypothetical protein (acidic stress response factor)
HP0547	87 (0,00005)	45	1	secreted	CagA, Cytotoxicity-associated immunodominant antigen (toxin transporter activity)
HP0072	114 (0,00005)	44	3	cytoplasm/secreted	UreB, Urease subunit beta (catalysis of urea degradation to ammonia and carbon dioxide)
HP0629	116 (0,00009)	26	7	cytoplasm	Uncharacterized protein
HP0294	80 (0,00046)	12	11	cytoplasm	AmiE, Aliphatic amidase, (Catalysis of the hydrolysis of short-chain aliphatic amides)
HP0389	87 (0,0008)	37	2	periplasm	SodB, Superoxide dismutase [Fe] (antioxidant)
HP1177	137 (0,00583)	6	6	outer membrane	HopQ, omp27
HP0570	92 (0,00617)	57	10	cytoplasm	PepA, Cytosol aminopeptidase (processing and regular turnover of intracellular proteins)
HP0243	72 (0.000775)	18	0	cytoplasm	NapA, DNA protection during starvation protein (DNA protection from oxidative damage)
HP0390	83 (0,01612)	31	2	cytoplasm/ periplasm	TagD, Probable thiol peroxidase (antioxidant)
HP0068	112 (0.01711)	9	3	cytoplasm	Urease accessory protein UreG (functional incorporation of the urease nickel metallocenter facilitation)
HP0875	75 (0,01743)	8	2	cytoplasm/ periplasm	KatA, catalase (hydrogen peroxide decomposition, oxidative stress response)
HP1555	111 (0,02063)	24	3	cytoplasm	Tsf, elongation factor Ts (translation elongation factor)
HP0824	93 (0,02114)	35	3	cytoplasm	TrxA, thioredoxin
HP0310	82 (0,02924)	9	0	cytoplasm	PgdA,Peptidoglycandeacetylase (N-deacetylation of peptidoglycan catalysis)
HP0025	172 (0,00005)	6	6	outer membrane	HopD (omp2)
HP0979	120 (0.05071)	12	2	cytoplasm	FtsZ, Cell division protein FtsZ (Essential cell division protein, procaryoticconterpart of tubulin)

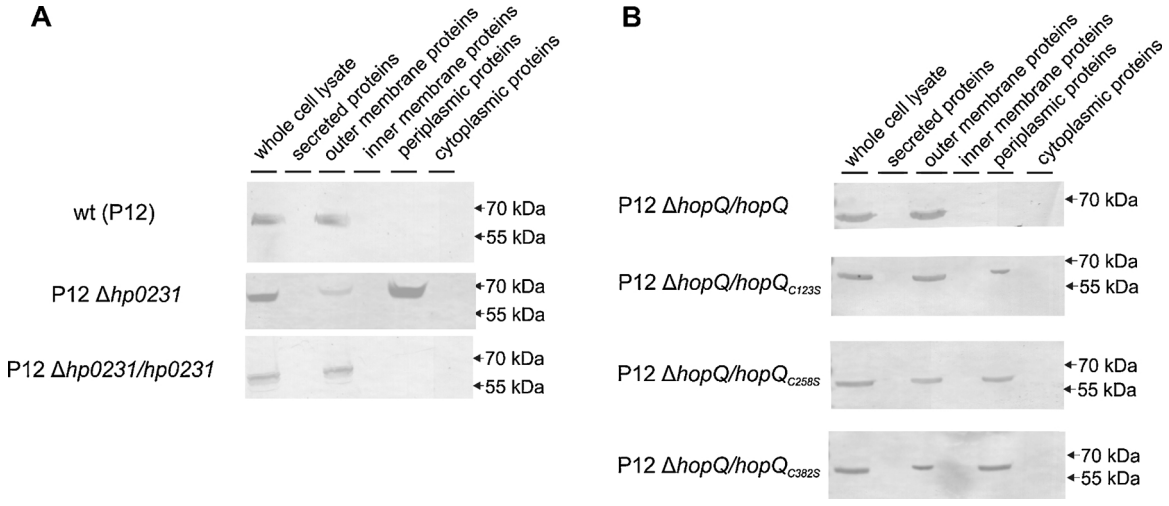

Fig. 4. Localization of HopQ within subcellular fractions of *H. pylori* strain P12wt, *H. pylori* strain P12 lacking HP0231, *H. pylori* strain P12 Δ*hp0231* and *H. pylori* strain P12 Δ*hp0231*/*hp0231* using anti-HopQ antibody (panel A).
Localization of HopQ mutated variants within subcellular fractions of *H. pylori* strain P12 with the complemented *hopQ* mutants: P12Δ*hopQ*/*hopQ*, P12Δ*hopQ*/*hopQ*$_{C123S}$, P12Δ*hopQ*/*hopQ*$_{C258S}$, P12Δ*hopQ*/*hopQ*$_{C382S}$ using anti-HopQ antibody (panel B).

adhesion interacting with the sialyl-Lewis^x antigen, as an HP0231 substrate, although it is an outer membrane-anchored and surface-exposed protein containing two disulfide bonds (Pang et al., 2014). The reason for this observation remains unclear. The structure of HopS (BabA) has been solved, and it was demonstrated that cysteine-clasped loop 2 (CL2) of HopS plays an important role in its binding to mucosal ABO/Le^b blood group carbohydrates (Hage et al., 2015; Moonens et al., 2016).

The objective of the next part of the research was to evaluate whether the cysteine residues determine the activity of HopQ. In this

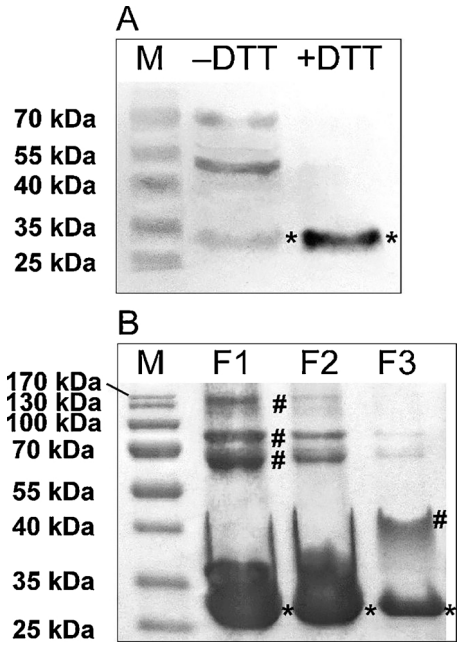

Fig. 5. Analysis of complexes between *H. pylori* 26695 HP0231-P258T and its protein substrates isolated using affinity chromatography (Ni-NTA agarose columns). A – Small scale experiment.Protein complexes, eluted from column were treated ornot-treated with DTT, separated by 12% SDS-PAGE and analyzed by immunoblot using anti-HP0231 antibodies; B – Large scale experiment.Protein complexes, eluted from the column were separated by 12% SDS-PAGE (non-reducing conditions) and visualized by Coomassie staining. Asteriskcorresponds to HP0231, and hash corresponds to complexes eluted from the columnand used for substrate identification by MS. F1, F2, F3 correspond to collected fractions.

work, we asked which disulfide bonds of HopQ are essential for its adherence onto gastric epithelial cells and for CagA delivery into host cells? *H. pylori* HopQ, whose structure has recently been solved, possesses six cysteine residues, namely Cys123, Cys152, Cys258, Cys290, Cys382 and Cys405, which generate three consecutive disulfide bridges (Javaheri et al., 2016). First, we determined the redox state of HopQ *in vivo* in *H. pylori* wt and *hp0231*-mutated strains by the AMS–trapping technique. AMS reagent moieties covalently react with reduced cysteines possessing a free thiol group –SH, resulting in a molecular mass increase of 490 Da/each cysteine. Oxidized dithiols are protected from this modification and, in consequence, the oxidized and reduced forms of proteins may be easily separated by non-reducing SDS-PAGE (Denoncin et al., 2013). We found that HopQ is present in the oxidized form in wild-type cells and in the reduced form in cells lacking HP0231, which suggests that it is actually a substrate of HP0231 (Fig. 6).

Next, the plasmid-encoded point-mutated versions of HopQ, in which a single cysteine residue was replaced with serine, were constructed. The insertion of the correct mutations was confirmed by sequencing, and recombinant plasmids were introduced into *H. pylori* lacking *hopQ*. First we analyzed the subcellular location of HopQ mutated versions. We found that HopQ mutated variants with only one defective disulfide bond, in contrast to wt HopQ, were present in both, outer-membrane fraction as well as in periplasmic compartment (Fig. 4B). Most of the HopQ of the *hp0231*-mutated strain was detected in the periplasmic compartment (Fig. 4A). Next, the resulting strains were used to check the activity of the mutated HopQ forms by comparing the T4SS-induced CagA translocation into AGS cells infected with *H. pylori*wt, *H. pylori* carrying a disrupted *hopQ* gene and *H. pylori* strains producing mutated HopQ variants. *H. pylori* wild type bacteria and a *hopQ* mutant complemented with a wild type version of *hopQ* induced a hummingbird phenotype, CagA phosphorylation and IL-8 production. Three variants of HopQ differed substantially in terms of analyzed characteristics. All phenotypic changes induced in AGS cells infected with the *H. pylori wt* was also created by AGS cell infection with *hopQ* mutant complemented with $HopQ_{C123S}$, whereas the $HopQ_{C382S}$ variant was unable to complement a *hopQ* mutant. $HopQ_{C382S}$ did not stimulate CagA phosphorylation and did not activate NF-κBto initiate IL-8 expression. Complementation of the lack of HopQ with $HopQ_{C258S}$ resulted in lower levels of CagA phosphorylation and IL-8 production (Figs. 7–9). These results showed that although HopQ contains six cysteine residues, only two are crucial for its function.

According to data presented by Javaheri et al., the outer membrane protein HopQ is the main *H. pylori* CEACAM binding adhesin. The HopQ-deficient *H. pylori* P12 strain show significantly reduced ability to adhere to cell lines expressing CEACAM1 (Javaheri et al., 2016). Thus, we next asked the question whether the observed effect of the lack of CagA translocation displayed by *H. pylori* expressing the $HopQ_{C382S}$ version results from its reduced level of interaction with the CEACEM1 receptor. We determined CEACAM1 binding properties of *H. pylori* P12 strains expressing wt HopQ, as well as its mutated versions, by pull down experiments. Interaction of analyzed strains with CEACAM1 was detected by Western-blots using anti-His tag antibodies. The control immunoblot developed with anti-HopQ serum documented that all used *H. pylori* strains produce comparable amounts of HopQ (Fig. 10). We showed that wt HopQ and its mutated version $HopQ_{C123S}$, bind strongly to CEACAM1 molecules, whereas the $HopQ_{C382S}$ version does not display an interaction with CEACAM1. The presence of $HopQ_{C258S}$ variant partially abolish *H. pylori* binding to CEACAM1 receptor. This documented that the disulfide bridge between Cys382 and Cys405 plays an essential role in the HopQ adhesion process.

It should be noted that comparative genomics performed on many *H. pylori* strains revealed a high level of genome diversity. Our work was done on two clinical isolates of *H. pylori*: *H. pylori* 26695 and *H. pylori* P12. OMPs of *H. pylori* 26695 strain have been recently precisely characterized (Voss et al., 2014). We documented that HP0231 affects CagA translocation by influencing disulfide bonds of HopQ. *HopQ* exists in two allelic forms: alleles I and II. The type I *hopQ* allele is often present in the genomes of highly pathogenic strains (Cao and Cover, 2002). Both investigated *H. pylori* strains contain HopQ type I (94% of amino-acid identity). We speculate that interactions between HP0231 and HopQ are rather universal among *H. pylori* isolates. It is rather independent on HopQ type as *hopQ* type II complements *hopQ* type I (see Belogolova et al). What is really controversial is the adhesion process which may be strain-specific. Although HopQ interaction with CEACAM receptors stimulates CagA translocation in the HP0231-dependent process, this interaction is not always strictly correlated with pathogen adhesion to gastric cells. Thus, expression of different adhesins by independent *H. pylori* strains may influence the bacterial binding and their function might be influenced by HP0231. Also the applied methodology and used cell line are of big importance (Loh et al., 2008; Belogolova et al., 2013; Javaheri et al., 2016). So to gain more precise insight into the process and to test the validity of the findings for the whole of *H. pylori*, more strains should be analyzed using various methodology.

4. Conclusions

HP0231, the main thioloxidoreductase of *H. pylori,* has an impact on pathogen virulence by influencing HopQ redox state, which facilitates translocation of CagA, the main pathogenic oncoprotein. However, HopQ is not the only significant target of HP0231 during interaction of *H. pylori* with epithelial cells. Also, other *H. pylori* outer membrane proteins, members of the Hop family, are potentially substrates of HP0231. These other proteins play various roles in *H. pylori* pathogenesis; for example, HopS or SabA are responsible for adhesion to various receptors and do not influence T4SS functioning. Our work on HP0231 substrate proteins also indicated other important virulence factors of *H. pylori*, such as catalase or GroEl, as substrates of the

Table 2
HP0231 protein substrates identified by a "reverse purification" substrate trapping technique and mass spectrometry. Protein bands that correspond to hypothetical complexes between HP0231 and its protein substrates were cut out from the gel. Next, protein complexes were digested and the obtained peptides were analyzed using mass spectrometry. Proteins with the highest scores are listed. Results were classified using UniProt ID, and their predicted localization and number of cysteines were checked. For selected proteins, their localization, based on experimental data, was also added. Those with scores in the control experiment ($HP0231_{CXXS}$) higher than 50% of value obtained in relevant experiment ($HP0231_{P/T}$) were rejected (Cao et al., 1998; Backert et al., 2005; Gonzalez-Lopez et al., 2013; Carlsohn et al., 2006; Davis et al., 2006; Voss et al., 2014; Ki et al., 2015; Zanotti and Cendron, 2014).

locus tag	gene name	uniprot ID	Protein name&function	score	number of cysteines	predicted localization	confirmed localization	references
HP_0010*	*groL*	CH60_HELPY	60 kDa chaperonin	12383	1	cytoplasm	cytoplasm/extracellular	(Cao et al., 1998; Backert et al., 2005; Gonzalez-Lopez et al., 2013)
HP_1430	*rnj*	RNJ_HELPY	Ribonuclease J	2217	3	cytoplasm	nt	
HP_1512*	*frpB-3*	O26042_HELPY	Nickel-regulated outer membrane protein (FrpB-3)	1426	3	cell outer membrane	cell outer membrane	(Carlsohn et al., 2006; Davis et al., 2006)
HP_1205	*tufA*	EFTU_HELPY	Elongation factor Tu	1339	2	cytoplasm	cytoplasm/ cell surface	(Backert et al., 2005)
HP_1177*	*omp27*	O25791_HELPY	Outer membrane protein HopQ	1300	6	cell outer membrane	cell outer membrane	(Carlsohn et al., 2006; Voss et al., 2014)
HP_0570*	*pepA*	AMPA_HELPY	Aminopeptidase	1188	10	cytoplasm	cytoplasm/extracellular	(Ki et al., 2015)
HP_1134	*atpA*	ATPA_HELPY	ATP synthase subunit alpha	1162	3	cell inner membrane	nt	
HP_0317*	*omp9*	O25086_HELPY	Outer membrane protein BabC (HopU)	589	8	cell outer membrane	cell outer membrane	(Carlsohn et al., 2006; Voss et al., 2014)
HP_1195	*fusA*	EFG_HELPY	Elongation factor G	540	6	cytoplasm	nt	(Backert et al., 2005)
HP_1243*	*omp28*	O25840_HELPY	Outer membrane protein BabA (HopS)	534	8	cell outer membrane	cell outer membrane	(Carlsohn et al., 2006; Voss et al., 2014)
HP_0025*	*omp2*	O24870_HELPY	Outer membrane protein HopD	490	6	cell outer membrane	cell outer membrane	
HP_0896*	*omp19*	O25556_HELPY	Outer membrane protein BabB (HopT)	409	6	cell outer membrane	cell outer membrane	
HP_0875*	*katA*	CATA_HELPY	Catalase	418	2	cytoplasm	cytoplasm/ extracellular	
HP_0227* /HP_1342*	*omp5 /omp29*	O34523_HELPY	Outer membrane protein HopM/HopN	337	4	cell outer membrane	cell outer membrane	

*Proteins classified as extracytoplasmic by Zanotti and Cendron (Zanotti and Cendron, 2014).

HP0231 thioloxidoreductase. Additionally, other candidates are the CagL and CagI proteins, which are involved in T4SS pilus formation (Backert and Blaser, 2016). Both contain two cysteine residues, and the residues in CagL generate a disulfide bridge (Pham et al., 2012; Barden et al., 2013). The role of disulfide bridges in CagL and CagI function has not been analyzed. However it was documented that CagI and CagL are reduced in levels in *H. pylori* strain lacking HP0231 (Zhong et al., 2016). The influence of the Dsb system on *H. pylori* virulence thus appears to be extremely complex.

Author contributions

The authors declare no conflict of interest.

EJ-K and MG conceived and designed the study. PR-J and AM prepared proteomic analysis, MG carried out majority of the laboratory work. KB-O, HS and AM carried out experiment aimed at HP0231 substrates detection. EJ-K, TM, RH and MG analyzed the data. EJ-K and MG wrote the manuscript.

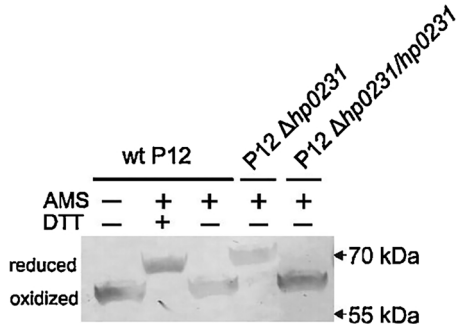

Fig. 6. Redox state of HopQ in wt, *hp0231::cat* mutated and *hp0231::cat* mutated complemented with *hp0231* strains of *H. pylori* P12 strains. Bacterial cultures were treated with 10% TCA, followed by alkylation with AMS. Cellular proteins, including reduced (DTT treated, modified with AMS) and oxidized (non-modified with AMS) controls, were separated by 14% SDS-PAGE under non-reducing conditions, and Western blot analysis using antibodies against HopQ was performed. Each lane contains proteins isolated from the same amount of bacteria.

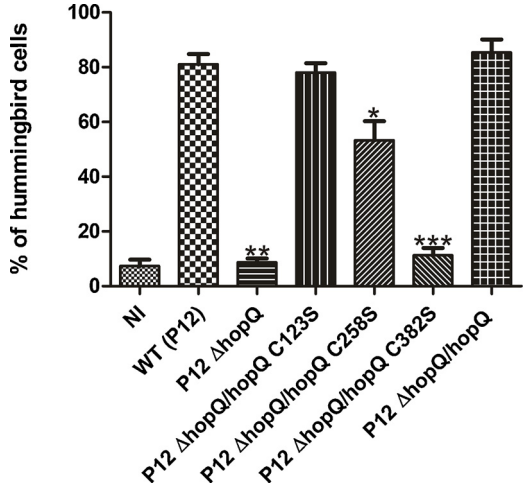

Fig. 7. Development of hummingbird phenotype in AGS cells upon infection with the *H. pylori* wild type strain P12 (WT), with its isogenic mutant P12Δ*hopQ*, and with the complemented hopQ mutants: P12Δ*hopQ/hopQ*, P12Δ*hopQ/hopQ*$_{C123S}$, P12Δ*hopQ/hopQ*$_{C258S}$, P12Δ*hopQ/hopQ*$_{C382S,}$ in comparison to non-infected AGS cells (NI). The results are the means of three independent experiments. The error bars indicate standard deviations. Statistical analysis was evaluated using Student's t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with WT P12 infected cells.

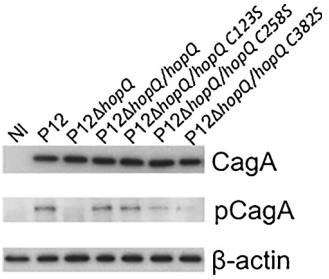

Fig. 8. CagA phosphorylation in AGS cells upon infection with *H. pylori* wild type strain P12 (WT), with its isogenic mutants P12ΔhopQ and with the complemented hopQ mutants: P12Δ*hopQ/hopQ*, P12Δ*hopQ/hopQ*$_{C123S}$, P12Δ*hopQ/hopQ*$_{C258S}$, P12Δ*hopQ/hopQ*$_{C382S,}$ in comparison with non-infected AGS cells (NI). Infections were performed at an MOI of 100 for 3 h. Phosphorylated CagA (pCagA), total amount of CagA (CagA) and AGS cells (β-actin) were estimated with specific antibodies by immunoblot analysis.

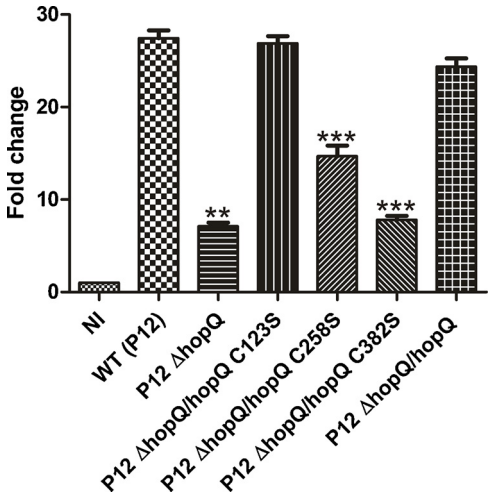

Fig. 9. Production of IL-8 in AGS cells upon infection with *H. pylori* wild type strain P12 (WT), with its isogenic mutants P12Δ*hopQ* and with the complemented hopQ mutants: P12Δ*hopQ/hopQ*, P12Δ*hopQ/hopQ*$_{C123S}$, P12Δ*hopQ/hopQ*$_{C258S}$, P12Δ*hopQ/hopQ*$_{C382S,}$ in comparison with non-infected AGS cells (NI) estimated by RT-PCR. Errors bars show ± SD of three independent experiments. Statistical analysis was evaluated using Student's t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with WT P12 infected cells.

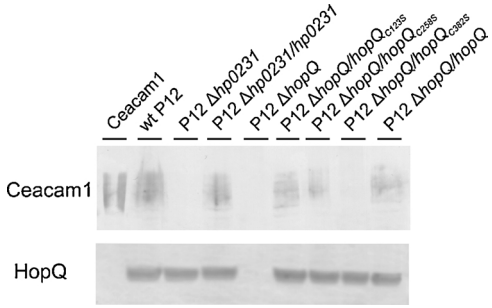

Fig. 10. Analysis of the *H. pylori* P12 wt and HopQ mutated variants (Δ*hopQ*, Δ*hopQ/hopQ*$_{C123S}$, Δ*hopQ/hopQ*$_{C258S}$, Δ*hopQ/hopQ*$_{C382S}$), binding to human CEACAM1 as determined after pull down experiments and detected by Western-blot (anti-His antibodies). Each lane contains proteins isolated from the same amount of bacteria. The protein bands are blurred because CECAM1 is highly glycosylated protein. As a control total amount of HopQ was estimated with specific antibodies by immunoblot analysis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.08.002.

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