Molecular architecture of *Streptococcus pneumoniae* surface thioredoxin-fold lipoproteins crucial for extracellular oxidative stress resistance and maintenance of virulence

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The respiratory pathogen *Streptococcus pneumoniae* has evolved efficient mechanisms to resist oxidative stress conditions and to displace other bacteria in the nasopharynx. Here we characterize at physiological, functional and structural levels two novel surface-exposed thioredoxin-family lipoproteins, Etrx1 and Etrx2. The impact of both Etrx proteins and their redox partner methionine sulfoxide reductase SpMsrAB2 on pneumococcal pathogenesis was assessed in mouse virulence studies and phagocytosis assays. The results demonstrate that loss of function of either both Etrx proteins or SpMsrAB2 dramatically attenuated pneumococcal virulence in the acute mouse pneumonia model and that Etrx proteins compensate each other. The deficiency of Etrx proteins or SpMsrAB2 further enhanced bacterial uptake by macrophages, and accelerated pneumococcal killing by H₂O₂ or free methionine sulfoxides (MetSO). Moreover, the absence of both Etrx redox pathways provokes an accumulation of oxidized SpMsrAB2 in vivo. Taken together our results reveal insights into the role of two extracellular electron pathways required for reduction of SpMsrAB2 and surface-exposed MetSO. Identification of this system and its target proteins paves the way for the design of novel antimicrobials.

INTRODUCTION

*Streptococcus pneumoniae* (pneumococci) are Gram-positive human commensals but also pathogens with high virulence potential. Pneumococci are dreaded as the etiologic agent of respiratory and life-threatening invasive diseases, such as pneumonia, meningitis and septicemia. The disease burden is high in developed and developing countries and, e.g. one million children under the age of 5 years are killed every year (Gomez & Hammerschmidt, 2012; Kadioglu et al, 2008). Its location in the human respiratory tract forces the bacterial cell to develop mechanisms to resist the host defenses like the oxidative burst produced by the innate immune system (West et al, 2011).

Reactive oxygen species (ROS) are also produced during one-electron transfer reactions to O₂ that include hydrogen peroxide...
et al, 1998; Zander et al, 1998).

Messens, 2010; Das & Das, 2000; Hanschmann et al, 2013; Kang et al, 2013). The oxidation of Met residues in proteins generates methionine sulfoxides (MetSO) that can induce conformational changes leading either to activation or inactivation of proteins (Zeller & Klug, 2011; Erickson et al, 2008) or in Escherichia coli the transcription factor HypT is activated specifically by MetSO formation (Drazic et al, 2013). This reaction is reversible and MetSO modifications can be reduced to Met by methionine sulfoxide reductases (Msr). Depending on the stereospecific orientation two distinct classes of Msr enzymes have been described. MsrA is specific for the reduction of the S-form and MsrB reduces the R-form of MetSO (Brot et al, 1981; Grimaud et al, 2001; Sharov & Schoneich, 2000; Weissbach et al, 2005). MsrA and MsrB occur typically as separate enzymes, but in some bacteria like S. pneumoniae, Neisseria gonorrhoeae and Haemophilus influenzae they are translationally fused as MsrAB (Delaye et al, 2007; Kim et al, 2009; Tarrago & Gladyshev, 2012; Wizemann et al, 1996). The reduction of MetSO by Msr involves the formation of an intramolecular disulphide in MsrA and MsrB, which is reduced by thioredoxins (Trx) that transfer electrons from their CXXC active site to the MsrA and MsrB disulphides (Hoshi & Heinemann, 2001; Lowther et al, 2000; Ranaivoson et al, 2009). The reduction of MsrA and MsrB by Trx leads to oxidation of Trx, which is reduced by the thioredoxin reductase (TrxB) on expense of NADPH. The Trx/TrxB system together with the glutaredoxin/glutathione (GSH)/GSH reductase system catalyse oxidative protein folding (Cho & Collet, 2013). However, also periplasmic reducing reductase pathways have been described in few pathogenic Gram-negative bacteria, such as in N. gonorrhoeae and N. meningitidis (Brot et al, 2006; Ranaivoson et al, 2006; Wu et al, 2005). In these periplasmic reducing reductase pathways of Gram-negative bacteria, the membrane-bound DsbD protein provides the electrons for reduction of sulfenic acids, for cytochrome maturation and to reduce periplasmic antioxidant enzymes, such as peroxidases and Msr (Cho & Collet, 2013; Denoncin & Collet, 2013). In pneumococci, the extracellular thioredoxin-like protein TlpA was suggested to be involved in the extracellular oxidative stress resistance, but DsbD-like reducing reductase pathways that are associated with TlpA were not explored (Andisi et al, 2012). In addition, the molecular interplay of the surface-exposed TlpA (renamed Etrx1) with its extracellular paralogue Etrx2 on oxidative stress resistance and virulence are unknown. This study discovers the unique molecular architecture of two surface-exposed thioredoxin-systems, Etrx1 and Etrx2 and their redox partners CcdA1, CcdA2 and SpMsrAB2 as important pneumococcal extracellular reducing systems essential for pneumococcal pathogenesis and oxidative stress resistance. We further provide evidence that both CcdA-Etrx pathways function in SpMsrAB2 reduction in vivo. Thus, Etrx1, Etrx2 and SpMsrAB2 are attractive targets for the design of novel anti-infectives to block the initial states of pneumococcal infection.

RESULTS

Identification and genetic organization of the etrx1 and etrx2 operons in pneumococci

The bioinformatic analysis of pneumococcal genomes identified two genes encoding surface-exposed thioredoxin-like lipoproteins, renamed here Etrx1 and Etrx2. Production of the Etrx proteins in S. pneumoniae D39 and other strains was demonstrated by immunoblot analysis (Fig 1). Etrx1 consists of 188 amino acids (20.8 kDa) and shows 39.4 % sequence identity with Etrx2 (Supporting Information Fig S1) that consists of 185 amino acids (20.7 kDa). The two potential reduct partners encoded by the conserved pneumococcal three-gene etrx1 operon are the methionine sulfoxide reductase AB2 (SpMsrAB2) and cytochrome C-type biogenesis protein, renamed here CcdA1 (Fig 1 and Supporting Information Figs S2–S5). SpMsrAB2 shows 77% of sequence homology with the intracellular pneumococcal SpMsrAB1 (Kim et al, 2009; Supporting Information Fig S6). The etrx2 gene is located together with a second ccdA-like gene (renamed ccdA2) in a bicistrionic operon, but without a second
Figure 1.
msrAB-like gene (Fig 1A and B and Supporting Information Figs S7 and S8). CcdA1 and CcdA2 share 58.8% sequence identity (Supporting Information Fig S9) and possess 18 and 21% sequence identity, respectively, with the transmembrane domain of the Neisseria periplasmic DsbD protein (Krupp et al, 2001; Supporting Information Fig S10).

Etrx1, Etrx2 and SpMsrAB2 are displayed on the pneumococcal surface and are essential for resistance against oxidative stress

Both Etrx proteins possess a typical lipoprotein-specific signal peptide containing a lipobox suggesting covalent binding of the putative lipoproteins to the outer leaflet of the phospholipid bilayer. The anchoring of lipoproteins is catalysed by a diacylglycerol transferase (Lgt), which adds a diacylglycerol residue to the thiol group of the conserved cysteine in the lipobox, while the signal peptide is cleaved after translocation and lipid-modification by the lipoprotein-specific signal peptidase Lsp. The lipid-modified cysteine residue remains at the mature lipoprotein as the new N-terminus (Kovacs-Simon et al, 2011). SpMsrAB2 contains one transmembrane segment and is probably anchored via this segment to the pneumococcal membrane. To analyse the surface localization of the putative lipoproteins Etrx1 and Etrx2 and of the membrane anchored protein SpMsrAB2, isoegenic mutants D39Δetrx1, D39Δetrx2 and D39ΔmsrAB2 were constructed by allelic replacement and verified by immunoblot analysis (Fig 1C and D). Flow cytometric analysis of wild-type and mutants demonstrated that Etrx1, Etrx2, and also SpMsrAB2 are displayed on the pneumococcal surface of wild-type bacteria, while no surface association of Etrx1, Etrx2 and SpMsrAB2 was found in the Δetrx1, Δetrx2 and ΔmsrAB2 mutants, respectively (Fig 2A). Proteolytic treatment of pneumococci with trypsin and pronase E, respectively, followed by immunoblot analysis confirmed these results (Supporting Information Fig S11). Immunoblot analysis was further conducted for the cytoplasmic and surface-displayed protein fractions of the wild-type and an lgt-mutant that is unable to anchor lipoproteins in the membrane (Voss et al, 2013). This subcellular analysis indicated that Etrx1 and Etrx2 are indeed lipoproteins and surface-exposed. They are released into the medium fraction of the lgt-mutant because of their inefficient anchoring to the membrane (Fig 2B). SpMsrAB2 is also displayed on the cell surface but has no lipid-anchor characteristic for lipoproteins and thus, is retained in the lgt-mutant membrane fraction (Fig 2B). However, we noticed that the SpMsrAB2 antibodies cross-reacted with the intracellular MsrAB1 that was detected in the cytoplasm of pneumococci (Figs 1D and 2B). Interestingly, the amount of SpMsrAB2 was enhanced in the ccdA1- and etrx1-mutants, which was not caused by the strategy of the mutant construction (Fig 1C and Supporting Information Fig S12).

To test the effect of Etrx lipoproteins and SpMsrAB2 on resistance against oxidative stress, D39 and its isogenic etrx-mutants were exposed for 30 min to 10, 15 and 20 mM H₂O₂ or for 90 min to 0.25, 0.5 and 0.75 mM paraquat (Fig 2C and D). The exposure to 10–20 mM H₂O₂ reduced the survival of the wild-type to about 50%. However, the etrx1/etrx2 double mutant was more affected and showed only 20–30% survival after exposure to 10 mM H₂O₂, and less than 2% survival in the presence of 20 mM H₂O₂. The survival of the etrx1 or etrx2 single mutants was also decreased by H₂O₂, but to a lower degree as determined for the double mutant (Fig 2C). Similar to the etrx-mutants, the survival of the msrAB2-mutant was affected by H₂O₂. Remarkably, the msrAB1-mutant was much more sensitive to H₂O₂ compared to the msrAB2 single or etrx double mutants (Fig 2C). This peroxide sensitive phenotype of the msrAB2-mutant is in agreement with a previous study (Andisi et al, 2012). The superoxide-generating compoud paraquat showed a weaker effect on the survival of the etrx single mutants (Fig 2D). Surprisingly, the etrx double and msrAB2 mutants were resistant to 0.25–0.5 mM paraquat, but the reasons are not known. The most dramatic effect on survival was determined for the msrAB1-mutant suggesting that SpMsrAB1 is more important for paraquat resistance than SpMsrAB2. These results suggest that both Etrx1 and Etrx2 can function as redox partners for SpMsrAB2 since the inactivation of both Etrx proteins or SpMsrAB2 renders pneumococci, to varying degrees, sensitive to extracellular peroxide stress.

Crystal structures of Etrx1 and Etrx2

The crystal structures of oxidized Etrx1 and Etrx2 have been solved in this study using the untagged-recombinant lipoproteins (Supporting Information Fig S13; details of the expression cloning and protein purification are described in the Supporting Information). Crystallographic data collection and model statistics are summarized in Table 1. The Etrx1 model comprises amino acid residues from Ala53 to Leu187. Etrx1...
presents a globular structure (38 × 28 × 29 Å) displaying a thioredoxin-like fold that contains seven β-strands and five α-helices (Fig 3A). Besides the canonical Trx fold, Etrx1 has two insertions (Supporting Information Fig S14). The first insertion (residues 53–72) results in b1, b2 and a1 elements, and the second insertion (residues 112–144) gives rise to an additional β-strand (b5) and a3 helix. The loop connecting β3 with a2 contains the CXXC motif (84CSIC87) defining the nucleophilic active site Cys84 and the resolving Cys87 (Fig 3D). These cysteine residues form a disulphide bridge reflecting the oxidized state of the protein. The structural data clearly show that only the active site Cys84 is solvent-exposed and accessible for electron transfer reactions. At the beginning of the second insertion and near the active site there is an additional loop connecting β4 with a3 (residues 112–120) that is not present in the closely related family of cytochrome maturation proteins. A search for proteins structurally related to Etrx1 performed with DALI server (Holm & Rosenstrom, 2010) identified the N-terminal domain of PilB protein (NterPilB) from N. gonorrhoeae (PDB code 2H30, Z score of 21.1 and rmsd of 1.5 Å for 133 Ca atoms), and N. meningitidis (PDB code 2FY6, Z score of 21.1 and rmsd of 2.1 Å for 136 Ca atoms) as the most closely related 3D structures. PilB is secreted to the periplasm and involved in the pathogen survival strategies against the oxidative burst as encountered in the host (Quinternet et al, 2008).

The three-dimensional structure of Etrx2 in complex with Cyclofos-3™ comprises the sequence from Ile43 to Asn185. Four molecules were found in the asymmetric unit presenting a similar structure (rmsd value of 0.184 Å for all Ca atoms) (Fig 3B). Etrx2 presents also a modified thioredoxin-like fold that

Figure 2. Etrx proteins are displayed on the pneumococcal surface and essential for oxidative stress resistance. Source data is available for this figure in the Supporting Information.

A. The surface localization of Etrx1, Etrx2 and MsrAB2 was analysed by flow cytometry. Wild-type pneumococci and isogenic mutants were incubated with anti-Etrx antibodies or PBS followed by detection using a goat anti-mouse IgG coupled Alexa-Fluor-488. The increase of fluorescence intensity (FL1-H) in the histograms indicates the presence of the Etrx and MsrAB2 proteins on the surface of non-encapsulated D39 bacteria, while the mutants and control treated pneumococci do not show an increase in fluorescence intensity.

B. Immunoblot analysis showing the surface localization of Etrx proteins and MsrAB2. Upper panel: pneumococci were fractionated and cytoplasmic (C) and cell membrane (M) fractions were separated by SDS–PAGE. Anti-Etrx or anti-MsrAB polyclonal antibodies were used to detect the proteins. Enolase was used as loading control and detected with anti-enolase antibodies (Hermans et al, 2006). Lower panel: Etrx1 and Etrx2 are lipoproteins as indicated by the absence of the protein in the isogenic lgt-mutant of D39cps (Δlgt) (Kovacs-Simon et al, 2011; Voss et al, 2013), while SpMsrAB2 is anchored in the membrane via one transmembrane domain (see also Fig 8 and Supporting Information S6).

C,D. Oxidative stress response. Encapsulated bioluminescent D39 and isogenic pneumococcal etrx-mutants were treated for 30 min with various concentrations of H2O2 (C) or 90 min with paraquat (D). Survival was determined by plating and determination of the CFU. Data are represented as mean ± SEM of three independent experiments.
is highly reminiscent of Etrx1 (rmsd value of 1.5 Å for 130 Ca atoms), where the classical thioredoxin-like motif of Etrx2 is embellished by a central αβ insertion and an N-terminal β-hairpin (Fig 3C). The CXXC motif (81CGPC84) defines the active site (Fig 3D). The main differences between Etrx1 and Etrx2 structures are the presence of eight extra residues forming a coil at the C-terminus of Etrx2, the conformation of β4–α3 loop, the disposition of α3 helix and the more extended conformation of the β7 strand in Etrx2 (Fig 3C and D). ResA, a thiol-disulfide oxidoreductase involved in cytochrome c biosynthesis in Bacillus subtilis and accepting electrons from CcdA, is the closest reported homologue of Etrx2 (rmsd value of 1.5 Å for 130 Ca atoms and Z score of 20.7). The Cyclofos-3™ is located in close proximity to the Etrx2 active site. The ligand, found in the four monomers of the asymmetric unit, is stabilized in a pocket (Fig 4A) formed by the β4–α3 loop, the α4 helix, the α4–β3 loop and the CXXC region (Fig 3B). This pocket presents a strong hydrophobic character (Ala78, Trp80, Ala109, Pro110, Ile112, Ala141, Phe144, Ile149, Ile122) versus the more polar character of the equivalent region in Etrx1 (Fig 4A–C). Cyclofos-3™ is stabilized by an H-bond with Gln113, by cation–π interaction of the polar face of the detergent with Trp80 and by π–π stacking interaction between phenyl ring of the detergent and Phe144 (Fig 4C). Attempts to obtain the reduced form of Etrx2 by co-crystallization with β-mercaptoethanol resulted in a new oxidized structure in which Etrx2 forms a complex with a 2-hydroxyethyl disulfide (HED) molecule (Fig 4D). A different conformation of the β4–α3 loop was observed coupled with the presence of the HED molecule at the hydrophobic cavity.

Both Etrx proteins share a conserved cis-proline residue (Pro156 in Etrx1 and Pro153 in Etrx2), placed in front of the catalytic cysteine residue, which is conserved in all thioredoxin-like proteins. As already mentioned above, the hydrophobic binding site, which is observed for Etrx2 but not for Etrx1, is of special interest. This hydrophobic binding site has not been observed in any extra-cytoplasmic thiol-disulfide oxidoreductases (TDORs) reported so far. Remarkably, both pockets differ in their amino acid composition and also in their conformation. Etrx2 presents a deep groove, while the Etrx1 site is filled by Tyr155 and covered by the α4–β3 loop. The electrostatic potential on the molecular surface is also quite different in both proteins. The Etrx2 binding site presents a hydrophobic character while Etrx1 presents a highly basic character in this region (Fig 4A). These differences point to a differential specificity of each protein for its redox partner.

### Functional analysis of the extracellular thioredoxin proteins Etrx1 and Etrx2 of *S. pneumoniae*

To decipher the function of Etrx1 and Etrx2 as thioredoxin proteins reducing the potential redox partner protein SpMsrAB2,
the redox states and potentials of purified Etrx proteins and SpMsrAB2 subunits (MsrA2 and MsrB2; Supporting Information Fig S13) were determined. Different ratios of GSH and glutathione disulfide (GSSG) were used to determine the redox potentials. The reduced thiols of Etrx1, Etrx2 MsrA2 and MsrB2 were alkylated with AMS in an anaerobic nitrogen environment, causing a mass shift after separation by SDS–PAGE. The redox potentials were calculated from densitometric analysis and the results revealed redox potentials of $-191 \pm 6$ mV for Etrx1, $-282 \pm 16.5$ mV for Etrx2, $-132.8 \pm 5.9$ mV for MsrA2 and $-120.9 \pm 0.6$ mV for MsrB2 (Fig 5A). The apparent redox potential of purified MsrAB2 has also been analysed and was determined between the redox potential of the individual MsrA2 and MsrB2 domains, confirming the previous results. Thus, thermodynamically, the transfer of electrons from both Etrx proteins to the Msr subunits of SpMsrAB2 would be possible. In order to elucidate whether both Etrx proteins can transfer electrons to SpMsrAB2 kinetically, the NADPH-dependent methionine sulfoxide reductase activity of rMsrA2 and rMsrB2, respectively, was measured in kinetic experiments with Etrx1 or

Figure 3. Three-dimensional structures of pneumococcal surface-exposed thioredoxins.
A. Overall Etrx1 structure showing $\beta$-strands as blue arrows and $\alpha$-helices as light blue helices. Catalytic Cys84 and Cys87 residues are labelled in red.
B. Overall Etrx2 structure representation showing $\beta$-strands as orange arrows and $\alpha$-helices in green. Cyclofos-3 is shown as sticks. Catalytic Cys81 and Cys84 residues are labelled in red.
C. Stereo view of the superimposition of Etrx1 (cyan) and Etrx2 (light brown). Loops around the active site are highlighted: the CXXC region in red, the $\beta4-\alpha3$ loop in yellow (Etrx1) and orange (Etrx2), the $\alpha4-\beta6$ loop in blue and the $\beta7-\alpha5$ in green.
D. Structural comparison between Etrx1 and Etrx2. Loops around the active site are highlighted: the CXXC region in red, the $\beta4-\alpha3$ loop in yellow (Etrx1) and orange (Etrx2), the $\alpha4-\beta6$ loop in blue and the $\beta7-\alpha5$ in green. Sequence alignment of Etrx1, Etrx2 and equivalent regions in NterPilB and ResA loop regions are shown for comparison. Composition and colours were chosen according to Quinternet et al (2009).
Figure 4. Structural differences between Etrx1 and Etrx2 active sites.

A. Electrostatic potential on the Etrx1 and Etrx2 molecular surface. The exposed active sites are marked with a black box. Catalytic cysteines are labelled. Acidic regions are coloured in red and basic regions in blue.

B. Stereo view of Etrx1 active site. Colour code as in Fig 3C.

C. Stereo view of Etrx2 active site. Relevant residues and Cyclofos-3 ligand are drawn as capped sticks. Carbon atoms of the ligand are shown in green, while those in the protein are colour coded as in Fig 3C. Hydrogen bonds are shown as dashed lines.

D. Stereo view of the Etrx2 catalytic core in complex with ligands. Shown is the superimposition of the reduced (light blue) and oxidized (light brown) forms. Relevant residues are labelled and shown as capped sticks. Polar interactions are shown as dashed lines.
Etrx2 as electron donor. Calculation of the specific activities indicated that Etrx1 efficiently regenerates oxidized MsrA2 but not MsrB2. In contrast, Etrx2 can reduce MsrB2 but has a 3.6-fold lower activity for the reduction of MsrA2 compared to Etrx1 (Fig 5B and Table 2).

The redox state of SpMsrAB2 was analysed in vivo for the ccdA single mutants as well as the ccdA1/2 and etrx1/2 double mutants using diagonal non-reducing/reducing SDS–PAGE assays followed by immunoblot analysis with anti-SpMsrAB2 and anti-pneumococcal antisera (Fig 5C). This diagonal assay distinguishes intramolecular and intermolecular disulphides in proteins (Leichert & Jakob, 2006). Bacterial extracts of non-encapsulated pneumococci were harvested under non-stress control conditions and proteins with reduced thiol-groups were irreversibly alkylated with NEM (N-ethylmaleimide), while disulphide bonds within the same protein and between different proteins are maintained. In this diagonal assay pneumococcal protein extracts were separated in the first dimension by non-reducing SDS–PAGE, the lane was cut and separated horizontally by a second reducing SDS–PAGE. Proteins with no disulphides run along the diagonal, while intramolecular disulphides migrate slightly above the diagonal. The diagonal immunoblot analysis revealed several SpMsrAB2 isoforms in the reduced form that were detected along the diagonal with

![Figure 5](image-url)
Table 2. Kinetic parameters of methionine sulfoxide reductase activity of MsrA2 or MsrB2

| Enzyme–substrate | V<sub>max</sub> (nmol/mg min) | K<sub>m</sub> (μmol/L) | Efficiency (V<sub>max</sub>/K<sub>m</sub>) |
|-------------------|-----------------------------|----------------------|----------------------------------|
| MsrA2 + Etrx1     | 59.0                        | 26.5                 | 2.22                             |
| MsrA2 + Etrx2     | 8.2                         | 13.3                 | 0.61                             |
| MsrB2 + Etrx1     | (No activity)               | (No activity)        | (No activity)                     |
| MsrB2 + Etrx2     | 17.1                        | 21.0                 | 0.81                             |

The methionine sulfoxide reductase activity was measured in the presence of Etrx1 and Etrx2 protein, respectively. The reaction was performed at pH 7.4 in a mixture containing the Etrx protein, NADPH, human thioredoxin reductase and the reaction was started by the addition of MsrAB2 subunits. AU, arbitrary units.

the anti-SpMsrAB2 antiserum (Fig 5C). The lower SpMsrAB2 isoform probably is mixed with SpMsrAB1 as shown by msrAB1- and msrAB2-mutant blots (Fig 5C; see also Fig 1D). Importantly, the oxidized intramolecular disulfide of the upper SpMsrAB2 isoform accumulates strongly above the diagonal in the D39ΔcpsΔcdaA1ΔcddA2 and D39ΔcpsΔetrx1Δetrx2 mutants (Fig 5C), but is only weakly detected in the single cdaA mutants. The results suggest that SpMsrAB2 is more oxidized in the cdaA1/2 and etrx1/2 double mutants compared to the single mutants. The functions of Etrx1 and Etrx2 as electron partners for SpMsrAB2 were further demonstrated by growth experiments in the presence of 6 mM free MetSO as physiological substrate. Growth in the presence of free MetSO was significantly impaired in the msrAB2 single or etrx1/2 and cdaA1/2 double mutants compared to the etrx or cdaA single mutants (Supporting Information Fig S15). This MetSO-sensitive phenotype is indicative for the deficiency of methionine sulfoxide reductase activity in the absence of both functional CcdA-Etrx electron pathways in vivo (Supporting Information Fig S15).

Etrx1 and Etrx2 are required for full virulence of pneumococci in an acute pneumonia mouse infection model

The acute experimental pneumonia and sepsis infection models were applied to assay the role of thioredoxin-like lipoproteins Etrx1 and Etrx2 on pneumococcal colonization and virulence in CD-1 outbred mice. In the acute pneumonia model mice (n = 12) were challenged intranasally with 1.0 × 10<sup>7</sup> bioluminescent wild-type D39lux or its isogenic mutants D39luxΔetrx1, D39luxΔetrx2 and D39luxΔetrx1Δetrx2, respectively. Mice infected with wild-type pneumococci or single etrx mutants showed the first weak signs of pneumococcal spread into the lungs at 30 h post-infection. In contrast, the D39luxΔetrx1Δetrx2 double mutant showed earliest at 72 h post-infection a strong increase in bioluminescence in the lungs as monitored by real-time bioimaging, which could be correlated with a strong increase in bacterial load in the lungs (Fig 6). This delay of pneumococcal pneumonia and sepsis after intranasal infection suggested an attenuation of virulence for the double mutant D39luxΔetrx1Δetrx2. In contrast, the single knockout mutants D39luxΔetrx1 and D39luxΔetrx2 showed no significant differences in bioluminescent flux compared to D39lux and had mostly developed severe lung infections or succumbed to sepsis 72 h post-infection (Fig 6D). The results of the real-time monitoring correlated with the survival rates of mice. The intranasal infection with the double mutant D39luxΔetrx1Δetrx2 prolonged significantly the survival time of mice (p < 0.0001), whereas survival of mice infected with the Etrx1- or Etrx2-deficient single mutants was not significantly altered compared to the wild-type
Figure 6.
infected mice (Fig 6A). Similar to the H$_2$O$_2$ resistance only the etrx double mutant is affected in virulence during in vivo infection, while in the study of Andisi et al. the attenuation of the mutant is due to the deficiency of Etrx1 (TlpA) and SpMsrAB (Andisi et al., 2012). To investigate whether the deficiency of SpMsrAB2 results in a phenotype similar to the double mutant D39luxΔetrx1Δetrx2, mice were also infected intranasally with the msrAB-mutants. Indeed, the results revealed significant attenuation of the msrAB2-mutant ($p < 0.0001$) compared to the wild-type D39lux (Fig 6B, E and F). Importantly, the etrx-mutants and the msrAB2-mutant showed no growth defects under in vitro conditions (Supporting Information Fig S16). In contrast, the deficiency of SpMsrAB1 impaired growth in a chemically defined medium (Supporting Information Fig S16) and pneumococcal virulence was also significantly reduced (Fig 6B) as has been shown previously (Wizemann et al., 1996). The real-time monitoring correlates with the survival rates of the mice (Fig 6E and F) and the lack of SpMsrAB1 and SpMsrAB2 had no additive effect. Furthermore, CD-1 mice ($n = 9$) were infected with a lower infection dose ($1 \times 10^6$) to explore the effect of Etrx proteins or SpMsrAB2 on nasopharyngeal colonization in the carriage model. Pneumococci were recovered after 1, 3 and 5 days from the nasopharynx and the lungs by broth inoculation. These results showed at all time points a significant reduction of nasopharyngeal carriage for the double mutant D39luxΔetrx1Δetrx2 compared to the wild-type (Supporting Information Fig S17). The other etrx- or msrAB2-mutants did not significantly differ in nasopharyngeal carriage from the isogenic wild-type (Supporting Information Fig S17). However, the msrAB2-mutant showed immediately at 24 h post-infection a significantly reduced number of CFU in the lung (Supporting Information Fig S17B).

To analyse the impact of the Etrx and SpMsrAB2 proteins on pneumococcal survival during sepsis, mice were infected via the intraperitoneal route. The survival rates of mice ($n = 12$) were similar for groups infected with wild-type or single etrx mutants, although there was a moderate but not significant attenuation for the double mutant D39luxΔetrx1Δetrx2 (Fig 6G). Similarly, the msrAB2-mutant showed no significant attenuation during invasive disease (Fig 6H).

Taken together, these in vivo infection experiments suggest that loss of one of the Etrx lipoproteins does not affect virulence. In contrast, loss of function of either surface-exposed Etrx lipoproteins or the SpMsrAB2 protein does significantly reduce virulence and spread of pneumococci from the nasopharynx into the lungs and blood.

### Pneumococcal resistance against killing phagocytosis relies on Etrx and SpMsrAB2

To investigate the role of the Etrx and SpMsrAB2 proteins on uptake by professional phagocytes and to allow significant phagocytosis of pneumococci we incubated the isogenic D39Δcps and individual etrx-mutants (Fig 7A). The number of recovered pneumococci was also significantly increased for the msrAB2-mutant (Fig 7A). The increased number of phagocytosed etrx double mutants or msrAB2-mutants was also confirmed by immunofluorescence microscopy (Fig 7B). Remarkably, the lack of Etrx1 in D39Δcps also accelerated phagocytosis (Fig 7A). In addition, the intracellular fate of wild-type and etrx-mutants was assessed. Regarding the intracellular survival, all strains showed a time-dependent decrease in the number of recovered and viable pneumococci. However, the relative decline of recovered D39ΔcpsΔetrx1Δetrx2 mutants did not show significant differences compared to the non-encapsulated S. pneumoniae D39Δcps. Similar to the non-encapsulated pneumococci, phagocytosis of the encapsulated strains showed a higher number of phagocytosed and recovered mutants deficient in both Etrx proteins. These data suggest that the total loss of Etrx lipoprotein or SpMsrAB2 activity accelerates phagocytosis and hence, killing of pneumococci.

### DISCUSSION

**Thioredoxin lipoproteins are required for resistance to extracellular oxidative stress**

In this study, we performed a comprehensive functional and structural analysis of the extracellular oxidative stress resistance system of S. pneumoniae mediated by two CcdA-Etrx pathways and their redox partner SpMsrAB2. Our results demonstrated that single etrx-mutants expressing SpMsrAB2 showed lower susceptibility to killing by H$_2$O$_2$, compared to the etrx double mutant that had a similar peroxide sensitive phenotype like the SpMsrAB2 mutant. Thus, only the deficiency of both Etrx1 and Etrx2 proteins or SpMsrAB2 significantly accelerated pneumococcal killing by H$_2$O$_2$ and diminished growth in the presence of MetSO, respectively. Similarly, only the deficiency of both, Etrx1 and Etrx2, or their electron acceptor SpMsrAB2 attenuates significantly pneumococcal virulence in the acute pneumonia model but not in the sepsis model. The importance of the Etrx proteins and SpMsrAB2 for virulence functions is supported by our phagocytosis assays, which showed that only
the uptake of the etrx double or msrAB2 mutant is massively enhanced, whereas only a minor effect was observed for the single etrx-mutants. These data suggest that the Etrx proteins can functionally replace each other, which ensures proper function of surface-exposed pneumococcal proteins. The determination of the redox potentials for the Etrx and SpMsrAB2 proteins together with our structural and kinetic data further demonstrate that SpMsrAB2 can be reduced by both Etrx proteins. Interestingly, Etrx1 seems to reduce preferentially the MsrA2 subunit of SpMsrAB2 while Etrx2 is able to reduce both MsrA2 and MsrB2 domains in vitro (Fig 5). Importantly, diagonal non-reducing/reducing SDS–PAGE analysis combined with SpMsrAB2 specific immunoblots further verified the in vivo accumulation of oxidized SpMsrAB2 protein especially in the ccdA1/2 and etrx1/2 double mutants. This indicates that both thioredoxin systems are required for efficient regeneration of SpMsrAB2. Interestingly, we detected strong growth sensitivities of the etrx1/2 double mutant in the presence of H2O2 and free MetSO as physiological MsrAB2 substrate. Hence, we postulate that both extracellular CcdA-Etrx-SpMsrAB2 electron pathways are involved in reduction of oxidized Met residues present in surface-exposed virulence proteins or as free MetSO on the bacterial surface that has to be further explored in future studies.

**Structural determinants of Etrx1 and Etrx2 proteins and evidence for two CcdA-Etrx-SpMsrAB2 pathways**

Thiol-disulfide oxidoreductases (TDORs) comprise a large superfamily of proteins that are present in all kingdoms of life where they control the redox state of Cys containing proteins. Cytoplasmic TDORs like the thioredoxin system are usually involved in maintaining protein cysteines in a reduced state (Fernandes & Holmgren, 2004). In contrast, periplasmic TDORs of Gram-negative bacteria like the Dsb family proteins or extracellular TDORs of Gram-positive bacteria catalyse disulphide bond formation or isomerization in the oxidizing periplasm or extracellular space (Cho & Collet, 2013; Denoncin & Collet, 2013). These extra-cytoplasmic or periplasmic TDORs are involved in a wide range of processes, including cytochrome maturation, e.g. B. subtilis ResA (Lewin et al., 2006), cell motility, e.g. E. coli DsbB (Dailey & Berg, 1993), natural competence development, e.g. B. subtilis DsbD (Meima et al., 2002), toxin biosynthesis, e.g. E. coli DsbA (Yamanaka et al., 1994) and synthesis of the endospore peptidoglycan cortex protective layer, e.g. B. subtilis StoA (Crow et al., 2009). Periplasmic reducing systems are present in this oxidizing compartment of Gram-negative bacteria to reduce sulfenic acids in the periplasm (e.g. DsbDC) or to deliver electrons for MetSO reduction (e.g. PilB) (Cho & Collet, 2013). Both Etrx1 and Etrx2 share sequence homology (30.48 and 21.83%, respectively) with the C-termini of the Dsb family proteins or as free MetSO on the bacterial surface that has to be further explored in future studies.

According to the accepted catalytic mechanism of thioredoxin-like proteins (Crow et al., 2004), Etrx1/Etrx2 would bind their redox partner SpMsrAB2 by means of a hydrophobic surface and subsequently perform a nucleophilic attack on the target disulphide bond via the N-terminal nucleophilic thiolate of the CXXC motif (Cys84 in Etrx1, Cys81 in Etrx2). This process would lead to the formation of a mixed intermolecular disulphide between Etrx and its redox partner proteins, which

![Figure 7. Influence of extracellular thioredoxin lipoproteins and MsrAB2 deficiency on uptake of S. pneumoniae D39Δcps by macrophages.](image-url)
is resolved by the second C-terminal resolving Cys residue (Cys87 in Etrx1, Cys84 in Etrx2). The N-terminal active-site Cys residue is present in the reactive thiolate anion form, which is stabilized by an interaction with the dipole of helix α2. In several thiol-disulphide oxidoreductases this interaction reduces the $pK_a$ of the nucleophilic active-site Cys by at least two pH units (Roos et al, 2013).

In the CXXC motif of both Etrx proteins, only the active-site Cys is solvent-exposed and accessible to the redox partner (Cys84 in Etrx1 and Cys81 in Etrx2). Many thioredoxin-like proteins share also a proline residue within the CXXC motif. This proline residue is also present in the CXXC motif of Etrx2 (Fig 3D), but not in Etrx1. The presence of proline residues at the cap of the active site helix has been reported to have important consequences for the distribution of the electrostatic field near the cysteines as proline does not possess a standard peptide group (Crow et al, 2009). The absence of proline in Etrx1 would affect the macrodipole arising from α2 helix that is often invoked as primary cause of the lowered $pK_a$ values associated with the active-site cysteine residue of the CXXC motif in most TDORs (Kortemme & Creighton, 1995). Furthermore, the limited conformational freedom of proline (in comparison with other residues) has been reported to be an important factor in maintaining rigidity of the CXXC motif and relevant for the structural changes from the reduced to its oxidized forms. Etrx1, representing one of the rare cases without proline in the CXXC motif, is therefore expected to have more structural variations between reduced and oxidized forms than Etrx2. Another relevant difference between both Etrx proteins and other extra-cytoplasmic TDORs concern a glutamate residue that is placed three positions after the C-terminal cysteine residue of the CXXC motif. Substitution of this glutamate has been shown to have a significant effect on the active site properties of ResA and StoA of B. subtilis (Crow et al, 2009; Hodson et al, 2008; Lewin et al, 2006). Etrx1 and Etrx2 do not have a glutamate at this position, but possess instead a serine residue (Ser90 in Etrx1 and Ser87 in Etrx2; Supporting Information Fig S18).

Etrx2 has an unprecedented hydrophobic cavity close to the active site. The presence of a hydrophobic patch near the active site has been associated with substrate recognition in other TDORs (Crow et al, 2009). In the structure of the oxidized forms of Etrx2, a detergent molecule (Cyclofos-3™) or a HED molecule is bound to this hydrophobic pocket, very likely mimicking the redox partner interaction. Etrx1 lacks this cavity and this region shows differences in both the nature of the amino acids and in the conformation of the β4–α3 loop (Fig 3). In agreement with these results, soaking experiments with Etrx1 crystals did not yield a complex with Cyclofos-3™ even at high concentrations of the detergent.

In conclusion, some of the structural determinants of the CXXC motif observed in other extra-cytoplasmic thio-redoxins, such as the presence of proline or glutamate residues are not observed in pneumococcal Etrx proteins (except for the proline in Etrx1). Despite strong similarities in the overall fold of both, Etrx1 and Etrx2, relevant differences are observed between their active sites (presence of hydrophobic cavity in Etrx2, different electrostatic potential, different CXXC motifs). These differences provide a structural basis for the specific interaction of Etrx with the MsrA or the MsrB domains of the SpMsrAB2 redox partner observed in the redox potential determination and kinetics.

**Model for the two CcdA-Etrx-SpMsrAB2 electron pathways**

The mechanism for the protection against oxidative stress via both CcdA-Etrx-SpMsrAB2 electron pathways is modeled in Fig 8 and Supplementary Information Movie 1. The pneumococcal cell wall is an oxidizing environment in which the sulphur-containing amino acids Met and cysteine are highly susceptible to oxidation by endogenously produced peroxide. Electrons are transported from the cytoplasmic NADPH pool to the cell wall to keep pneumococcal surface proteins in a reduced state. The first proteins of this extracellular electron transport system are the integral membrane proteins CcdA1 and CcdA2. Electrons from the cytoplasmic Trx are shuttled between CcdA1 and CcdA2 to Etrx1 and Etrx2, respectively, similarly to that observed between the transmembrane and the periplasmic domains of DsbD. Surface-exposed Etrx1 and Etrx2 deliver electrons to the SpMsrAB2 protein for the reduction of MsrA2 (by Etrx1 or Etrx2) and MsrB2 (by Etrx2) domains (Fig 8). Oxidation of Met results in a mixture of the two diastereomers methionine-S-sulfoxide and methionine-R-sulfoxide, which are reduced by MsrA and MsrB, respectively. Besides the catalytic domains, SpMsrAB2 also carries a transmembrane segment that anchors the protein to the membrane. It has also a long and flexible coiled coil region allowing the enzyme to reach damaged virulence proteins and to reduce MetSO (Fig 8 and Supporting Information movie). Thioredoxin-like lipoproteins Etrx1/Etrx2 are critical in the turnover of the system by reducing the methionine sulfoxide reductase SpMsrAB2. Since this seems to be the sole extracellular thioredoxin system of pneumococci, the lack of functional Etrx proteins or SpMsrAB2 protein has direct consequences to resist oxidative stress and host immune defense mechanisms. In this sense, the pneumococcal surface-exposed thioredoxin systems reported here provide an important framework for the development of new antibacterial therapies.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions and transformation techniques**

E. coli strains and S. pneumoniae genotypes and strains used in this study are listed in Supporting Information Table S1. E. coli and S. pneumoniae strains were cultured and transformed as described recently (Jensch et al, 2010).

**Primers, construction of pneumococcal mutants and protein purification**

Primers that were used in this study and plasmids used for the mutagenesis and recombinant protein expression are listed in Supporting Information Table S2. For the generation of the pneumococcal mutants in D39lux (Jensch et al, 2010) and D39Δcps the insertion–deletion mutagenesis strategy was used as described (Rennemeier et al, 2007). Mutagenesis, expression cloning and protein
Figure 8. Proposed mechanism of oxidative stress defense mediated by the CcdA1-Etrx1 and CcdA2-Etrx2 electron pathways and their redox partner SpMsrAB2 on the pneumococcal surface. Electrons provided from NADPH by the cytoplasmic TrxB (SPD_1287) are transferred to the cytoplasmic TrxA (SPD_1567) and shuttled between the integral membrane protein CcdA1 (SPD_0571) to the surface-exposed thioredoxin-like Etrx1 (SPD_0572), and between the integral membrane protein CcdA2 (SPD_0885) to surface-exposed thioredoxin-like Etrx2 (SPD_0886) following the same mechanism. Both thioredoxin-like proteins provide reducing equivalents to SpMsrAB2 (SPD_0573) for the reduction of the MsrA (by Etrx1 or Etrx2) and MsrB (by Etrx2) domains. SpMsrAB2 remains anchored to the membrane and presents a long and flexible coiled coil region allowing SpMsrAB2 to reach and repair ROS damaged surface proteins. Catalytic cysteine residues in Etrx1, Etrx2 and SpMsrAB2 are represented as green spheres. Experimentally determined redox potentials for each protein are labelled. White arrows indicate presumed interactions and grey arrows indicate those demonstrated experimentally. All accession numbers refer to the S. pneumoniae D39 annotation.
The paper explained

PROBLEM:
The respiratory pathogen *Streptococcus pneumoniae* (the pneumococcus) is a serious pathogen causing life-threatening community-acquired pneumonia and invasive diseases. The high morbidity and mortality caused by pneumococcal diseases (more than 1.5 million every year, particularly in infants, elderly and immunocompromised patients), is exacerbated by the increasing prevalence of antibiotic-resistant strains and the suboptimal efficacy of available vaccines. Pneumococci have evolved efficient mechanisms to resist protein damage under oxidative stress conditions and to displace other bacteria in the nasopharynx. While oxidative stress-resistance mechanisms in the cytoplasm are well studied, the extracellular mechanism required to resist attack from the host is less investigated.

RESULTS:
We have identified a two-operon system responsible for the extracellular oxidative stress resistance. This system is composed of two integral membrane proteins (CcdA1 and CcdA2), two thioredoxin-like lipoproteins (Etrx1 and Etrx2) and a single methionine sulfoxide reductase (SpMsrAB2). We have solved the crystal structures of both Etrx proteins and analysed the functions of both Etrx and SpMsrAB2 proteins on oxidative stress resistance and virulence. We further observed in phagocytosis experiments with macrophages that both thioredoxin lipoproteins and SpMsrAB2 play a crucial role in pneumococcal pathogenesis. We can finally conclude that both Etrx proteins function as electron donors for the SpMsrAB2 redox partner and are therefore crucial for the extracellular reducing redox pathways of pneumococci.

IMPACT:
The data highlight the crucial role of thioredoxin lipoproteins Etrx1 and Etrx2 and SpMsrAB2 for virulence and the redox-reactions of the extracellular oxidative stress resistance mechanism of pneumococci. Suppression of that system severely reduces pneumococcal virulence and lethality. In this sense, the combined effect of antibiotics with new ligands blocking this crucial pneumococcal system could be intended. Therefore, our data provide a new framework for the development of novel bactericidal agents against this important human pathogen.

purification of His₆-tagged proteins are described in detail in the Supporting Information.

Pneumococcal survival under oxidative conditions
The survival experiments under oxidative condition with hydrogen peroxide (H₂O₂), paraquat (stimulating superoxide production in cells; kindly provided by A. Littmann, Julius Kühn Institute (JKI), Braunschweig, Germany) or MetSO (Sigma-Aldrich, Taufkirchen, Germany) were conducted as described previously (Johnston et al, 2004). Briefly, wild-type pneumococci and Etrx-deficient mutants were cultured in THY broth at 37°C to mid-log phase and treated for 30 min with 10, 15 or 20 mM H₂O₂ and 90 min with 0.25, 0.5 or 0.75 mM paraquat, respectively. Untreated pneumococci were used as a control. To determine the percentage of survival, serial dilutions were plated onto blood agar plates and CFU were counted after overnight incubation at 37°C and 5% CO₂. In control experiments catalase (5000 U/ml) was added simultaneously with H₂O₂ to the bacterial cultures.

Protein crystallization
Etrx1 crystals were obtained with 30% v/v PEG 4000; 0.1 M tris pH 8.5; 0.2 M MgCl₂ 18°C. Etrx2: Cyclofos 3™ complex crystal was obtained with 3.4 M sodium malonate pH 6.0 18°C, while the Etrx2:HED Complex crystal was obtained in 30% PEG 15000 supplemented with 14 mM β-mercaptoethanol. Details in Supporting Information.

Data collection, phasing and model refinement
Native data sets of Etrx1 and Etrx2: Cyclofos-3™ crystals were collected on ESRF ID14-4 beamline in Grenoble, France. Native data set of Etrx2:HED crystal was collected on SLS PXIII beamline in Villigen, Switzerland (Table 1). Details in Supporting Information.

SpMsrAB2 homology model
The 3D model for the catalytic domains of pneumococcal SpMsrAB2 (residues 60–312) was obtained by comparative homology via modeler and energy minimization.

Ethics statement
Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). All experiments were approved by the ethical board and Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLFV MV), Rostock in Germany (permit no. 72213.1-1.006/09 and 72213.1-1.019/11).

Mouse models of infection and bioluminescent optical imaging
Eight weeks old female outbred CD1 mice (Charles River, Sulzfeld, Germany) were infected intranasally or intraperitoneally with bioluminescent pneumococci as described recently (Hartel et al, 2011; Jensch et al, 2010). Briefly, pneumococci were cultured to A₅₆₀ = 0.35 in THY supplemented with 10% foetal bovine serum and the infection dose was adjusted to 1.0 × 10⁷ CFU in 25 μl for the intranasal route (n = 12) and 5 × 10⁵ CFU in 100 μl for the intraperitoneal route (n = 12). Before intranasal infection, mice were anaesthetized by intraperitoneal injection of ketamine (Ketanest S; Pfizer Pharma, Karsluhe, Germany) and xylazine (Rompun®; Provet AG, Lyssach, Germany). Once anaesthetized the animals were scuffed, with the nose held upright, and the bacterial suspension of 25 μl was administered intranasally by adding a series of small droplets into the nostrils for the mice to involuntarily inhale. The infection dose was confirmed by determination of the CFU after plating serial dilutions...
of the infection dose on blood agar plates. Bioluminescent optical imaging using the IVIS® Spectrum Imaging System (Caliper Life Sciences, Hopkinton, US) allowed monitoring of pneumococcal dissemination after intranasal infection (Hartel et al, 2011; Jensch et al, 2010). At pre-chosen time intervals post-infection mice were imaged for 1 min to monitor dissemination of pneumococci into the lungs. A time series of the images was generated and the bioluminescent intensity (BLI) was determined by quantification of the total photon emission using the LivingImage® 4.1 software package (Caliper Life Sciences).

Phagocytosis experiments
To determine the rate of phagocytosed wild-type and mutant pneumococci and their intracellular survival in macrophages, phagocytosis experiments with j774A.1 murine macrophages (DSMZ, Braunschweig, Germany) were carried out as described (Hartel et al, 2011; Jensch et al, 2010).

Statistical analysis
All data are reported as mean ± SD unless otherwise noted. Results were statistically analysed using the unpaired, two-tailed Student’s t-test. Kaplan–Meier survival curves were compared by the log rank test. p Values for bioluminescence measurements were calculated using the unpaired, one-tailed t-test for differences between groups, while differences of one group between days were analysed by the paired t-test. Statistical significance was confirmed by ANOVA analysis with Bonferroni’s multiple comparison post hoc test. A p-value < 0.05 was considered to be statistically significant.

For more detailed materials and methods see the Supporting Information.

Accession numbers
Sequence data for the etrX genes 1 and 2 of D39 or TIGR4 are deposited in the EMBL/GenBank databases under accession numbers ABJ55360 and ABJ55355 or AAK74804 and AAK75117. Sequence data for the ccaA1 and ccaA2 of D39 or TIGR4 are deposited in the EMBL/GenBank databases under accession numbers ABJ54003 and ABJ54567 or AAK747803 and AAK75116. Sequence data for the msrA2B gene of D39 or TIGR4 are available from the EMBL/GenBank databases under accession numbers ABJ53896 or AAK74805. The atomic coordinates and structure factors for Etrx1, Etrx2:Cyclofos™ complex and Etrx2:HED complex (codes 4HQ5, 2YP6 and 4HQZ, respectively) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, US (http://www.rcsb.org/).

Author contributions
MS, JAH, CHL, HA and SH conceived and designed the experiments. MS, SGB, MRA, IJ, TMA, LP, TP and MG performed the experiments. MS, SGB, MRA, TP, JAH, CHL, HA and SH analysed the data. MS, SGB, HA, JAH and SH wrote and reviewed the paper.

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Supporting Information is available at EMBO Molecular Medicine Online.

The authors declare that they have no conflict of interest.

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