A genome-wide chromatin-associated nuclear peroxiredoxin from the malaria parasite

*Plasmodium falciparum*

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Malaria parasites are subjected to high levels of oxidative stress during their development inside erythrocytes and the parasite’s ability to defend itself against this assault is critical to its survival. Therefore, *Plasmodium* possesses an effective antioxidant defense system that could potentially be used as a target for the development of inhibitor based therapy. We have identified an unusual peroxiredoxin protein that localizes to the nucleus of *Plasmodium falciparum* and have renamed it PfnPrx (PF10_0268, earlier called MCP1). Our work reveals that PfnPrx has a broad specificity of substrate being able to utilize thioredoxin and glutaredoxin as reductants and having the ability to reduce simple and complex peroxides. Intriguingly, chromatin immunoprecipitation followed by deep sequencing reveals that the enzyme associates with chromatin in a genome-wide manner with a slight enrichment in coding regions. Our results represent the first description of a dedicated chromatin-associated peroxiredoxin and potentially represent an ingenious way by which the parasite can survive the highly oxidative environment within its human host.

Malaria is one of the world’s most common infectious diseases with approximately 500 million cases each year and one to three million deaths (1). The disease has an enormous burden on human health and causes decreased productivity and economic growth, and increased poverty (2). The lack of an effective vaccine, the emergence of resistance to first-line drugs such as chloroquine and antifolates and recent reports of clinical cases of reduced susceptibility to artemisinin in Cambodia (reviewed in (3)), combined with the small number of suitable new drugs against the malaria parasite demonstrate the need to identify potential new targets.

The malaria-infected erythrocyte is under constant attack from reactive oxygen and nitrogen species (ROS and RNS respectively). These are produced exogenously by the host immune system in response to infection, or endogenously by the generation of redox-active by-products resulting from the high metabolic rates of the multiplying parasite and the degradation of large quantities of haemoglobin (4). The ability of *P. falciparum* to protect itself against oxidative damage is thus critical and the parasite possesses multiple biochemical pathways able to mediate antioxidant defence and redox regulation (5,6). *P. falciparum* lacks catalase and a genuine glutathione peroxidase and therefore relies heavily on peroxiredoxins for the reduction of ROS and RNS (7).

Peroxiredoxins (Prx) are a family of thiol-dependent peroxidases known to reduce and scavenge hydroperoxides and thus form an important part of the cellular antioxidant machinery. Most peroxiredoxins contain at least one cysteine residue in their active site and because of its ability to react with the peroxide substrate it is referred to as the peroxidatic cysteine. This residue is the defining feature of this class of enzymes also referred to as AhpC-TSA family (8) The reaction results in the

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formation of a sulfenic acid intermediate that is usually reduced by the resolving cysteine present in the 2-Cys peroxiredoxins. The inter- or intramolecular disulfide bond formed can be reduced by thioredoxin, glutaredoxin or glutathione depending on the substrate specificity of the respective peroxiredoxin. For instance, mammalian 2-Cys peroxiredoxin 1 can only react with thioredoxin and does not accept glutaredoxin or thioredoxin as substrates (9,10).

Apart from their role as antioxidants, the proteins are also known to function as redox sensors in response to oxidative stress. They have also been implicated in cellular processes such as apoptosis, cell proliferation and differentiation (11). This functional diversity can be attributed to their structural flexibility, which leads to the association of the dimers to high M_r oligomeric proteins or vice versa, the susceptibility of one of their active site cysteines to hyperoxidation by their peroxide substrates and their high abundance in the cell (12). In addition it was shown that the phosphorylation of peroxiredoxins affects their subcellular distribution and enzymatic activity (13).

Usually organisms contain more than one peroxiredoxin, which are found in different subcellular locations such as cytosol, mitochondria, peroxisomes and nucleus (8). This is also the case for *Plasmodium*. To date, five malarial peroxiredoxins have been characterized: cytosolic PfTrx-Px1 and mitochondrial PfTrx-Px2 (14-16), cytosolic PfTrx-Px3 (17,18), PfAOP, potentially localizing to the apicoplast (19) and finally a glutathione-dependent-like peroxidase with a preference for thioredoxin as reducing substrate (7). *P. falciparum* also possesses several proteins belonging to the thioredoxin superfamily which provide reducing equivalents to the peroxiredoxins (reviewed in (6)).

In addition to the five characterized peroxiredoxins the malaria parasite possesses an additional protein with a conserved AhpC-TSA domain called Merozoite Capping Protein-1 (MCP1, PF10_0268) (20,21). MCP-1 was described as a cytosolic protein localizing to the invasion tight junction, a zone of tight apposition between the merozoite and the red blood cell membrane formed during the invasion process (20). However, it is not known if the AhpC-TSA domain of MCP-1 is functional (20,21). Additionally, MCP-1 has two other defined domains with a negatively charged middle region enriched in glutamate and a C-terminal positively charged section enriched in lysine. It was speculated from the solubility properties of MCP-1 and the C-terminal lysine-rich domain that this region may be required for binding to the cytoskeleton within the invading merozoite (21).

In this work, we show that MCP1 is a peroxiredoxin with unusual biochemical characteristics. Since this enzyme exclusively localizes to the nucleus (in contrast to previous reports) we renamed it PfnPrx (*Plasmodium falciparum* nuclear peroxiredoxin). In addition, we demonstrate that PfnPrx is associated with the parasite’s chromatin in a genome-wide manner suggesting a potentially essential role in the protection of nuclear DNA against oxidative stress.

**EXPERIMENTAL PROCEDURES**

*Parasite cultures:* *P. falciparum* asexual stage parasites were maintained in human erythrocytes (blood group O+) at a hematocrit of 4% with 0.5% (w/v) Albumax™ (Invitrogen) (22). *P. falciparum* 3D7 parasites were originally obtained from David Walliker at Edinburgh University. Cultures were synchronised as previously described (23).

*Antisera:* Rabbit and mouse antibodies were generated against PfnPrx1-100, containing the conserved oxido-reductase domain, from a GST fusion protein expressed from a plasmid construct using the following primers: PfnPrxF (5’- CGCGGATCC ATGGCTCAATTAGCAGAAAATAC-3’); and PfnPrxR (5’- CCGCGATTC ATGGCTCAATTAGCAGAAAATAC-3’); and PfnPrxR (5’- CGCGGATTC ATGGCTCAATTAGCAGAAAATAC-3’). PCR products were digested with *Bam*H1/*Xho*1 (underlined letters in primers), purified and cloned into the plasmid pGEX4T-1 (Amersham Biosciences). For immunoblots, saponin-lysed parasite pellets from highly synchronous 3D7 parasites were separated in sample buffer on 4-12% (w/v) SDS-NuPAGE gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (Schleicher & Schuell). Affinity purified PfnPrx rabbit anti-
serum and anti-PfnPrx mouse monoclonal were
diluted in 0.1% (v/v) Tween 20-phosphate-
buffered saline with 1% (w/v) skim milk.
Appropriate secondary antibodies were used,
and immunoblots were developed by ECL
(Amersham Biosciences). For the time-course of
expression analysis, proteins extracted from an
equal number of cells were used for each time
point.

Fluorescence Imaging: Fluorescence images of
parasites were captured using a Carl Zeiss
Axioskop microscope with a PCO Sensicam
and Axiovision 2 software. For immunofluorescence
assays of free and/or invading merozoites, highly
synchronous schizont stage 3D7 parasites in the
process of rupture/reinvasion were smeared and
fixed in 100% methanol at –20°C. After
blocking in 3% (w/v) bovine serum albumin
(Sigma) the cells were incubated for 1 hour with
the appropriate antisera: rabbit anti-PfRON4
(1/200) (24,25), mouse monoclonal anti-PfnPrx
(1/200). Bound antibodies were then visualised
with Alexa Fluor-488/594 anti-rabbit IgG or
anti-mouse IgG (Molecular Probes) diluted
1:1000. Parasites were mounted in Vectashield
(Vecta Laboratories) containing DAPI (4',6-
diamidino-2-phenylindole; Roche Molecular
Biochemicals).

Immunoelectron Microscopy: Parasites for
electron microscopy immunolabeling were fixed
and prepared as described previously (26). The
primary antibody used was the rabbit anti-
PfnPrx. Samples were washed and incubated
with secondary antibodies conjugated to 15-nm
colloidal gold (BB International). Samples were
then post-stained with 2% aqueous uranyl-
acetate then 5% triple lead and observed at 120
kV on a Philips CM120 BioTWIN Transmission
Electron Microscope.

Vector construction, transfection and Southern
Blotting: To create the plasmid used for the
integration of GFP at the 3’end of the nPrx gene
by single cross-over, a PCR fragment containing
exon 3 of nPrx without a stop codon was cloned
into the Bgl II-Avr II sites of the pARL1-GFP
vector (27,28). Parasites were transfected and
integrants were selected as previously described
(27). Integration was monitored by Southern
blots according to standard procedures.

Protein purification and Enzymatic Assays: A
PCR fragment containing nucleotides 1 to 492 of
PfnPrx was amplified from P. falciparum cDNA
and cloned in the Bam H1-Xho 1 site of the pET-
45b expression vector (Novagen). The latter
directs expression of the protein with an N-
terminus (His)6 tag to facilitate purification of the
recombinant protein. Generation of the
nPrxC56S mutant was performed by site-
directed mutagenesis with the Quickchange II
XL kit from Agilent Technologies. Induction
and purification of 6-His-nPrx1,164 were
performed according to standard procedures. P.
falciparum TrxR and PfTrx1 were generated as
described (29,30). P. falciparum glutaredoxin 1
(PfGrx1, PFC0271c) was amplified from 3D7
genomic DNA and cloned in the Bam H1-Xho
1 site of the pET-45b expression vector
(Novagen). Induction of the protein was
performed at 27°C overnight.

The overall antioxidant activity of
PfnPrx was estimated by analyzing its ability to
protect E. coli glutamine synthetase (Sigma)
from inactivation by a thiol-metal catalysed
oxidation system (DTT/Fe3+/O2) (31). The P.
falciparum nPrx kinetic parameters were
determined (30) using a spectrophotometric
assay to determine the initial rates of the
peroxidase reaction as previously described for
Toxoplasma gondii Trx-Px2 (30,32). For the
assays using the thioredoxin system the reaction
mix contained 100 mM HEPES pH 7.6, 1 mM
EDTA, 0.25 units P. falciparum thioredoxin
reductase, 2 µM PfnPrx, 200 µM NADPH,
varying concentrations of P. falciparum
cytosolic thioredoxin PfTrx1 (20–100 µM) at a
constant concentration of 20 µM hydrogen
peroxide.

For assays using the GSH system with
P. falciparum Grx1, the reaction mix contained
100 mM HEPES pH 7.6, 1 mM EDTA, 0.5 units
glutathione reductase, 1 mM GSH, 2 µM PfnPrx,
200 µM NADPH, varying concentrations of P.
falciparum cytosolic glutaredoxin PfGrx1 (1-30
µM) at a constant concentration of 20 µM
hydrogen peroxide or varying concentrations of
hydrogen peroxide (1–50 µM) and cumene
hydroperoxide (1–100 µM) at a constant
concentration of 10 µM PfGrx1. No NADPH
oxidation was observed without addition of
PfGrx1 indicating that PfnPrx cannot be reduced
by GSH itself. The decrease in absorbance at 340 nm due to NADPH oxidation was determined over 30 sec with time points taken every 10 msec. Assays were performed at 25°C. The linear rates were determined and fitted to the Michaelis–Menten equation using Graphit 5.0 (Erithracus).

Chromatin immunoprecipitation and deep sequencing. Chromatin immunoprecipitation of nPrx-GFP from formaldehyde crosslinked and sonicated chromatin was performed as described (33) using an anti-GFP antibody (AbCam 290). Immunoprecipitated as well as input chromatin was treated at 45°C in the presence of 0.5 M NaCl and tested by qPCR (for primer list see supplementary table 1) or used for Illumina sequencing library preparation according to the linear amplification protocol (34). Sequencing libraries were loaded on the Illumina Genome Analyzer IIx and sequenced for 36 cycles from one side of the fragments. Quality filtered 35 bp sequence reads were mapped against the P. falciparum genome assembly (PlasmobD v6.1) using the standard Illumina pipeline. Coverage plots were generated using uniquely mapped sequence reads by counting the number of overlapping tags in 100 bp windows and visualized in SignalMap (NimbleGen). The ratio track was obtained by dividing the ChIP-seq tag counts with the input tag counts and displayed on a log2 scale. For generation of average gene profile the coding body of all “normal size” genes (1-10Kb) were divided into twenty equal size windows and five 150 bp windows immediately up- or downstream represented flanking sequences. The ratios of the tag counts in the ChIP vs input dataset has been computed in each individual windows and averaged in the corresponding window of all genes. For scatter plot analysis the ratios of ChIP and input tag counts have been calculated in the coding body of each gene have been plotted against the RNAseq tag density (tag/1000 bp transcript) of an independent 3D7 schizont population (34).

RESULTS

Identification of PfnPrx (earlier MCP1) as a nuclear peroxiredoxin. To investigate the role of PfnPrx (MCP1) in the merozoite invasion process we generated an antibody against the conserved oxidoreductase domain (Fig. 1A) (20). Western blots on schizont stage protein extracts showed a single specific band of approximately 55 kDa, which was slightly higher than the predicted 45 kDa (Fig 1B, 48 hours lane) and is in agreement with previous results (20). All the other P. falciparum peroxiredoxins having sizes between 22 and 28 kDa, we can be confident that our antibody is not cross-reacting with any of them. We next performed a time course analysis for expression of PfnPrx (MCP1) using parasite protein extracts taken at 8 hr intervals throughout the erythrocytic asexual cycle. The PfnPrx (MCP1) protein was present as a single 55 kDa band in all samples analysed with the highest expression between 32 to 48 hr (Fig 1B) which corresponds to the schizont stage where the parasite undergoes nuclear division and also when proteins involved in merozoite invasion are expressed. This pattern is also in agreement with the RNA expression analyses previously published (35).

To determine the subcellular localisation of the PfnPrx (MCP1) protein we performed immunofluorescence assays on parasites from different stages of the erythrocytic cycle. The anti-nPrx (MCP1) monoclonal antibody overlapped almost completely with the DAPI stained parasite DNA in rings, trophozoites and schizonts suggesting that PfnPrx was located in the nucleus (Fig. 2A) a subcellular localisation different to that described previously (20). Analysis of the protein sequence with the PredictProtein software (36) also revealed a potential nuclear localisation signal in the C-terminus of PfnPrx (KKPAKKKVKKKK). To analyse the subcellular localisation of PfnPrx (MCP1) in more detail we performed IFA experiments on merozoites during erythrocyte invasion in which we observed that PfnPrx also localised to the parasite’s nucleus. No anti-PfnPrx (MCP1) signal was found at the tight junction, visualised by staining with an antibody against the RON4 protein, a well characterised marker of this transient structure (25). To determine whether this localisation was due to our antibody recognising a protein other than PfnPrx (MCP1) we engineered a parasite line in which the green fluorescent protein (GFP) gene
was integrated by single crossover at the 3’ end of the *PfnPrx* (*MCP1*) gene leading to the production of a fluorescent *PfnPrx* (*MCP1*) (Fig. 3A and B). Western blot analysis showed that *PfnPrx* (*MCP1*) was detected as an 80 kDa protein with both the *PfnPrx* (*MCP1*) mouse monoclonal antibody or an anti-GFP monoclonal antibody confirming expression of the chimeric protein and providing additional evidence for the specificity of our mouse monoclonal anti-nPrx antibody (Fig. 3C). To determine the localisation of the *PfnPrx*-GFP fusion, the 3D *PfnPrx*-GFP line was analysed by epifluorescence microscopy. As shown in Figure 3D, the *PfnPrx*-GFP fluorescence overlaps with the DAPI stained parasite DNA in rings, trophozoites and schizonts confirming that nPrx (*MCP1*) is found in the nucleus of the parasite throughout the blood stage cycle. To determine more precisely the localisation of *PfnPrx* in the nucleus we performed immuno-electron microscopy on *P. falciparum* schizont stage sections. *PfnPrx* localises predominantly to the more electron dense regions at the nuclear periphery and this is believed to represent heterochromatin (Fig. 2C)(37). Consequently, because of the nuclear localisation and functional data presented below we renamed this protein *PfnPrx* (*P. falciparum* nuclear peroxiredoxin).

*PfnPrx* is a peroxiredoxin with unusual characteristics. *PfnPrx* possesses an N-terminal conserved AhpC-TSA domain. To investigate its functionality the N-terminal portion of the protein (amino acids 1 to 164) was recombinantly expressed and purified (Fig. 4A) and its potential peroxidase activity was assessed using various enzymatic assay systems. Initially we determined whether the protein protects glutamine synthetase from inactivation by a thiol-metal catalysed oxidation system (DTT/Fe^{3+}/O_{2}) (31). 4.5 µg of the recombinant peroxiredoxin-like domain of *PfnPrx* rescues glutamine synthetase (GS) activity by 50 % (Fig. 4B) confirming that the AhpC-TSA domain was catalytically active therefore suggesting that *PfnPrx* has antioxidant capacity. The specificity of this catalytic activity was further investigated by exchanging the predicted active-site cysteine residue at position 56 by serine, which totally abolished the GS protection activity of the recombinant protein (Fig. 1A and Fig. 4B). The fact that the recombinant AhpC-TSA domain of *PfnPrx* showed the ability to protect GS activity is presumably attributable to its capacity to reduce hydrogen peroxide. This suggests that this part of the protein acts as a peroxidase that might potentially protects nuclear components such as DNA from oxidative insults. To identify its natural reducing substrate, the catalytic activity of *PfnPrx* was analysed in the presence of *P. falciparum* thioredoxin 1, glutathione and glutaredoxin 1. *PfnPrx* had the ability to reduce hydrogen peroxide and this activity increased with increasing concentrations of PfTrx1 without reaching saturation at concentrations as high as 100 µM (Supp Fig. 1) suggesting that is most likely not the enzyme’s favoured reductant.

In addition to the thioredoxin system, *P. falciparum* possesses a functional glutathione system and we studied the possibility that it could be involved in the reduction of *PfnPrx*. No *PfnPrx* peroxidase activity on hydrogen peroxide was detected when using GSH as a reductant while addition of glutaredoxin 1 (*PfGrx1*, (15)) to the assay mix supported the reduction of *PfnPrx* with saturation kinetics (Fig. 4C) and a reasonable reaction rate. At a concentration of 20 µM H_{2}O_{2}, the apparent K_{m} for *PfGrx1* was determined to be around 4 µM showing that it was an excellent reductant for *PfnPrx*. When looking at the affinity of *PfnPrx* for H_{2}O_{2}, an apparent K_{m} of 14 µM was calculated when 10 µM of *PfGrx1* was used (Fig. 4D), demonstrating that H_{2}O_{2} was a good substrate for *PfnPrx* and corroborating the results obtained with the GS protection assay. In addition, *PfnPrx* was able to reduce cumene hydroperoxide with an apparent K_{m} of around 30 µM (Fig. 4E). The specific activity of the recombinant protein was in the range of 6 µmol/min/mg protein which is low compared to the activity of other *Plasmodium* peroxiredoxins (16,30). It cannot be excluded that regulatory or structural features important to define substrate specificity are missing from the recombinant *PfnPrx* given that only the AhpC-TSA domain of *PfnPrx* rather than the full-length protein was analysed.

As *PfnPrx* is localised to the nucleus, it would be expected that its favoured physiological reductant should also localise to this organelle or at least have the ability to translocate to it from the cytoplasm upon
oxidative stress. To investigate the cellular localisation of PfGrx1 we generated parasites expressing a PfGrx1-GFP fusion. PfGrx1-GFP mostly localises to the cytoplasm of the parasite though some of protein overlaps with the DAPI stained nuclear DNA suggesting that it has the ability to translocate between the two compartments (Supp Fig. 2). However, we cannot at this stage exclude that the nuclear staining is the result of a mislocalization of the fusion protein due to its overexpression from an episome.

Having demonstrated that PfnPrx had the ability to detoxify peroxides *in vitro* we hypothesised that it could potentially be involved in protecting the parasite’s nucleus from oxidative stress. However, it has not been possible to demonstrate a consistent increase in the level of PfnPrx transcript or protein when submitting the parasites to exogenous oxidative stress *in vitro* using a glucose/glucose oxidase system (not shown).

**PfnPrx is potentially essential in the erythrocytic stage.** To gain further insight into the role of PfnPrx in the blood stage cycle of the malaria parasite we attempted to generate a knock-out line in both *P. falciparum* and *P. berghei*, a rodent malaria parasite often used as a model for human malaria due to the ease with which knock-out strains can be generated. Despite several attempts we were unable to generate a nPrx knock-out line in either of the *Plasmodium* species tested. This was in contrast with the ability to easily tag the PfnPrx with GFP suggesting that the protein performs an essential/important function in the erythrocytic stage of the malaria parasite (not shown).

**ChIP-seq analysis reveals genome-wide binding of PfnPrx to chromatin.** The co-localisation of PfnPrx with the DAPI stained DNA suggested that it was potentially associating with chromatin. To investigate this possibility we performed a genome-wide ChIP-seq analysis on schizont stage 3D7nPrx-GFP parasites using an anti-GFP antibody. Intriguingly, visual inspection of the data showed nearly even distribution of PfnPrx across the *P. falciparum* genome (Fig. 5A). An obvious exception was the strong depletion of PfnPrx from the centromeric regions (Fig 5B). In addition, PfnPrx was found to be slightly enriched in the coding body of genes (Fig 5B), which was also apparent on the average gene profile (Fig 5C). Enrichment of PfnPrx in coding regions and its depletion from centromeres were confirmed by ChIP-qPCR, and were found to be consistent across all stages of intraerythrocytic development (Fig. 5D). Importantly, recoveries with the GFP antibody for chromatin isolated from non-tagged 3D7 parasites were very low for all sites tested proving the specificity of our assay (data not shown). Finally, we determined whether enrichment of PfnPrx showed a correlation with transcriptional activity. As demonstrated by scatter plot analysis (Fig. 5E) PfnPrx occupancy in coding regions showed limited variation across genes and no or minimal correlation with steady state mRNA levels. Notably, outlier genes with high PfnPrx occupancy tended to be tRNA or rRNA genes. In conclusion, the ChIPseq data demonstrates the intimate association of PfnPrx’s with the malaria genome.

To determine if the association of PfnPrx with the chromatin was mediated through interactions with other proteins we performed immunoprecipitation on the 3D7nPrx-GFP line using an anti-GFP antibody. Mass spectrometry analysis revealed, in addition to nPrx-GFP, a high number of different proteins such as core histones, heat shock proteins, proteasome components and a few transcription factors however none with very high peptide coverage (Fig. 6). This high variety of proteins was somewhat expected based on the scale of nPrx’s broad association with the genome and the absence of any clear specific interactor suggests that nPrx might interact directly with the chromatin, potentially through its highly positively charged C-terminus as has been shown for proteins like Linker Histone H1 (38).

**DISCUSSION**

MCP-1 was previously described as a protein localising within the cytoplasm of the merozoite and associating with the moving junction as the parasite invades the host erythrocyte (20,21). It was noted that this protein contained an oxidoreductase domain although it had not been demonstrated to be functional. In contrast to the
previous observation we here show that MCP-1 is exclusively localised within the nucleus of \textit{P. falciparum}. Moreover we demonstrate that the protein contains a functional peroxiredoxin domain with unique characteristics. Consequently, we have renamed the protein PfnPrx. The functional properties of PfnPrx as a peroxiredoxin, its subcellular localisation within the nucleus, its coverage of a large proportion of \textit{P. falciparum} chromosomes and our inability to inactivate its gene in two different species of \textit{Plasmodia} suggest that it plays an important role in this compartment.

Peroxiredoxins are a family of antioxidants that protect the cell from metabolically produced reactive oxygen species. The \textit{P. falciparum} PfnPrx prefers glutaredoxin over thioredoxin as a reducing substrate and shows saturation kinetics. The protein reduces hydrogen peroxide and cumene hydroperoxide equally well suggesting that it does not discriminate between anorganic or organic hydroperoxides. Its specificity for glutaredoxin as a reducing substrate is unusual especially because most other known peroxiredoxins or peroxiredoxin-like proteins react with thioredoxin or glutathione. Peroxiredoxins with this substrate specificity have so far only been described in plants although some of these can also be reduced by thioredoxins (9,39,40) (and reviewed in (41)). Intriguingly, PfnPrx was associated strongly with the parasite’s genome suggesting that the protein has a role in either protecting nuclear components such as DNA from oxidative damage or being involved in maintaining chromatin structure, DNA repair or mechanisms that affect transcriptional activities. In fact it has been shown that redox active thiol-containing proteins and peptides are critical for these processes because they can respond to oxidation or reduction processes that allows for the regulation of their activities (42). There are a number of reports that show interaction of human peroxiredoxin 1 with the nucleus. This binding capacity and the downstream effects on transcriptional regulation can be either dependent or independent of the peroxidase activity of the protein and it has been suggested that apart from acting as an antioxidant the protein can act as a chaperone to stabilise other DNA-binding factors (43-45).

The broad distribution of PfnPrx across the \textit{P. falciparum} genome suggests that it plays a general role across the chromosomes and it could possibly be associated with nucleosomes. Studies on humans, \textit{Drosophila} and worms have shown that nucleosome occupancy increases in coding regions and that this is independent of transcriptional status (46-48). In addition, our previous studies on core histone H3 demonstrated a slight enrichment in the coding body of genes similar to that observed for PfnPrx. Such similar enrichment profile is consistent with a potential association of PfnPrx with nucleosomes (49). Due its very highly positively charged C-terminus, PfnPrx could potentially interact directly with the phosphate backbone of the DNA, without the need to link through an interacting partner. It is indeed well documented that the lysine-rich C-terminal tails of linker histone H1s bind directly to the linker DNA entering and exiting a nucleosome leading to the formation of higher-order folding states of chromatin (50,51). While linker histones of multicellular eukaryotes display a tripartite structure made up of a conserved globular domain flanked by two less structured N- and C-terminal domains, their counterparts in several protists and bacteria only contain the lysine-rich C-terminus (reviewed in (52)). It is worth mentioning that although H1-like basic proteins have been found in dinoflagellates (53) and ciliates (54), none have been identified for any of the apicomplexans, also members of the alveolates family. It is tempting to speculate that the lysine-rich C-terminus of PfnPrx could potentially play a similar role in chromatin structure however our attempts to recombinantly express the full length PfnPrx or its C-terminal tail to investigate a potential association with nucleosomes \textit{in vitro} have unfortunately been unsuccessful so far. In any case, if PfnPrx is involved in the protection of the genome against oxidative stress, the pairing of a peroxiredoxin domain with a highly basic domain suggests an ingenious means of making sure that the enzyme would always be in a position to quickly get rid of any oxidative insult as soon as it is produced therefore limiting the chances of DNA damage.

Moreover, we were unable to disrupt the PfnPrx gene either in \textit{P. falciparum} or \textit{P. berghei} suggesting that it plays an essential role...
for nuclear integrity or function possibly through protecting nuclear DNA and chromatin-binding, thiol-containing proteins from oxidative damage or because it is intimately involved in the regulation of gene expression. In conclusion, we have identified a dedicated nuclear peroxiredoxin with broad substrate specificity that associates with chromatin throughout the genome potentially demonstrating a specific adaptation of the malaria parasite to its hostile environment.

REFERENCES

42. Go, Y. M., and Jones, D. P. Antioxid. Redox Signal 13, 489-509

FOOTNOTES
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**FIGURE LEGENDS**

**Fig. 1.** PfnPrx is expressed throughout the erythrocytic stage. A) Domain organisation of PfnPrx PF10_0268. The alignment shows the conserved AhpC-Tsa family domain with the peroxidatic cysteine. Comparison was realised with Praline software. The Genbank accession numbers of the aligned sequences are: PfnPrx, AAC46600; *S. cerevisiae* DOT5, P40553; PfTPx1, XP_001348542; PfTPx2, XP_001350554; Pf1-Cys_Prx, AAG14353; PfAOP, AY306209. The asterisks represent conserved residues and the arrow highlights the peroxidatic cysteine. B) Time-course of PfnPrx expression during the blood stage using an affinity-purified rabbit anti-serum raised against the first 100 residues of PfnPrx.

**Fig. 2.** PfnPrx is a nuclear protein. A) Immuno-fluorescence using the mouse monoclonal anti-PfnPrx antibody demonstrates localisation of PfnPrx in the nucleus throughout the erythrocytic stage. B) PfnPrx does not localise to the tight junction of merozoites in the process of invasion. Immuno-fluorescence with mouse monoclonal anti-nPrx and rabbit anti-RON4 antibody shows that PfnPrx is restricted to the nucleus in parasites invading a red blood cell. C) Immunoelectron microscopy using the rabbit anti-PfnPrx antibody on schizont stage parasites reveals that PfnPrx is in the electron dense nuclear periphery. The image shows a single merozoite located within a schizont.

**Fig. 3.** Endogenously tagging nPrx confirms its status as a nuclear protein. A) Schematic of the strategy employed to generate the 3D7 nPrx-3’GFP line. B) Southern blots confirming integration of the targeting construct at the endogenous nPrx locus. C) Western blots using the mouse monoclonal anti-nPrx or an anti-GFP antibody confirms that the 3D7nPrx-3’GFP parasite line expresses the nPrx-GFP chimera. D) Epifluorescence microscopy on the 3D7nPrx-3’GFP line demonstrates that the tagged nPrx localises to the nucleus in all blood stages.

**Fig. 4.** PfnPrx is a peroxiredoxin with unusual characteristics. A) Purification of the N-terminal domain of PfnPrx B) Glutamine synthetase protection assays revealing that PfnPrx is an antioxidant protein and that this activity was dependent on its conserved peroxidatic cysteine. C, D and E) Peroxidase assays showing that C) glutaredoxin is a potent reductant of PfnPrx and that PfnPrx efficiently reduces D) hydrogen peroxide and E) cumene hydroperoxide. 2 μM PfnPrx was used for all assays, 20 μM hydrogen peroxide was used for the variable Pf glutaredoxin 1 assay and 10 μM PfGrx1 was used for the variable peroxide assays. Results are representative of at least three independent experiments.

**Fig. 5.** PfnPrx shows genome wide chromatin association (A-B) Broad distribution of PfnPrx (A), its enrichment in coding and depletion at centromeric region (B) demonstrated by coverage plots and ratio track of PfnPrx-GFP ChIP-seq data obtained from schizont stage parasites. C) Average gene profile of PfnPrx occupancy. D) qPCR data confirming enrichment of PfnPrx in coding region and its depletion at centromeres at three different stages of intraerythrocytic development. E) Scatter plot analysis of correlation between PfnPrx enrichment in coding body of genes and steady state mRNA levels (RNA-seq data of schizont stage parasites from Bartfai et al, see earlier)
Fig. 6. PfnPrx-GFP associates with a broad range of proteins. The immunoprecipitated proteins were grouped in categories and plotted according the total number of peptides identified. HSPs: Heat shock proteins; RNA/DNA: Proteins involved in RNA/DNA metabolism and/or binding. The list of proteins with accession numbers is available in the supplementary table 1. No GFP peptides were recovered because a *Plasmodium falciparum* specific database was used for peptide identification.
Fig 1, Richard et al 2010
A genome-wide chromatin-associated nuclear peroxiredoxin from the malaria parasite *Plasmodium falciparum*

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