REVIEW ARTICLE

Iron metabolism in trypanosomatids, and its crucial role in infection

M. C. TAYLOR* *and* J. M. KELLY

Pathogen Molecular Biology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

(*Received 1 October 2009; revised 17 October 2009; accepted 18 November 2009; first published online 15 February 2010*)

SUMMARY

Iron is almost ubiquitous in living organisms due to the utility of its redox chemistry. It is also dangerous as it can catalyse the formation of reactive free radicals – a classical double-edged sword. In this review, we examine the uptake and usage of iron by trypanosomatids and discuss how modulation of host iron metabolism plays an important role in the protective response. Trypanosomatids require iron for crucial processes including DNA replication, antioxidant defence, mitochondrial respiration, synthesis of the modified base J and, in African trypanosomes, the alternative oxidase. The source of iron varies between species. Bloodstream-form African trypanosomes acquire iron from their host by uptake of transferrin, and *Leishmania amazonensis* expresses a ZIP family cation transporter in the plasma membrane. In other trypanosomatids, iron uptake has been poorly characterized. Iron-withholding responses by the host can be a major determinant of disease outcome. Their role in trypanosomatid infections is becoming apparent. For example, the cytosolic sequestration properties of NRAMP1, confer resistance against leishmaniasis. Conversely, cytoplasmic sequestration of iron may be favourable rather than detrimental to *Trypanosoma cruzi*. The central role of iron in both parasite metabolism and the host response is attracting interest as a possible point of therapeutic intervention.

Key words: *Trypanosoma*, *Leishmania*, iron transport, transferrin, anaemia, superoxide dismutase.

INTRODUCTION: THE IMPORTANCE OF IRON

Iron is the fourth most common element in the Earth's crust. It is present in the vast majority of living organisms and is the most abundant transition metal in the human body (Halliwell and Gutteridge, 2007). The biological utility of iron stems from its redox chemistry, which allows it to catalyse multiple types of electron transfer reactions. Iron generally exists in 2 oxidation states, Fe (II) and Fe (III), but may also be found as Fe (IV), Fe (V) or Fe (VI). The very ability of iron readily to undergo oxidation/reduction cycles also leads to its inherent toxicity as a catalyst for the production of reactive chemical species, such as the hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

This dangerous property requires that organisms which utilize iron maintain very tight control over its transport, metabolism and storage.

* Corresponding author: Tel: 0044 207 927 2615. Fax: 0044 207 636 8739. E-mail: martin.c.taylor@lshtm.ac.uk

The balance between the need for iron and its toxicity is especially apparent during an infection (Schaible and Kaufmann, 2004; Radtke and O'Riordan, 2006). Here, the host must fulfil its own iron requirements, whilst blocking the needs of the pathogen. In addition, the host may utilize iron-mediated radical production as part of its own immune armament, but must minimize harm to itself. Mammals have evolved an elaborate control system to sequester iron away from invading pathogens, whilst pathogens in turn have evolved multiple and varied methods to obtain it (Schaible and Kaufmann, 2004). The parasitic trypanosomatids of mammals face different challenges in their quest for iron, as they inhabit different niches within the host. In the following sections, we review current knowledge of the ways that *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* obtain iron from their mammalian host, and describe the multiple roles that iron plays within these parasites. We discuss the effect of infection on host iron metabolism and the role of iron homeostasis in pathogenesis. Finally, we assess the therapeutic possibilities of interfering with parasite iron metabolism.

doi:10.1017/S0031182009991880

IRON UPTAKE AND TRANSPORT

Iron uptake and transport in the host

In mammals, most iron is found in erythrocytes bound to haemoglobin. The majority of this is effectively recycled by macrophages during erythrophagocytosis. However, there is inevitably some loss and iron must be replenished from the diet, either as free iron or as haem. Dietary iron is usually in the form of Fe^{3+} and this must be reduced prior to transport across the duodenal epithelium. In the bloodstream, iron is transported as Fe^{3+} bound to the carrier glycoprotein transferrin. Mammalian cells express transferrin receptors (TfR1 and TfR2), which are internalised on binding of holotransferrin. The receptor-transferrin complex is trafficked into the endosomal system. As the sorting endosome matures, it becomes acidified by the vacuolar ATPase, and Fe^{3+} is released from transferrin as the pH drops. The transferrin is then recycled to the cell surface along with its receptor, where it is released to re-enter the plasma. Fe^{3+} is essentially insoluble at physiological temperature and pH, so transport across the endosomal membrane and into the cytoplasm requires that it be reduced to Fe^{2+} by a ferric reductase. Fe^{2+} is then transported by one of a number of divalent cation transporters (e.g. DMT1, also known as NRAMP2/Slc11a2) into the cytoplasm.

When intracellular iron levels are high, cytoplasmic Fe^{3+} is sequestered in the storage protein ferritin and transferrin uptake is reduced. Ferritin and transferrin receptor levels are inversely regulated by the iron regulatory proteins IRP1 (cytoplasmic aconitase) and IRP2 to ensure that cellular iron overload or starvation is avoided (Wallander *et al.* 2006). IRP1 acts as a sensor of iron levels through its iron-sulphur cluster, which is required for its aconitase activity. When intracellular iron levels decrease, the FeS cluster disassembles, causing a conformational change and exposing the RNA binding domain (Dupuy *et al.* 2006; Walden *et al.* 2006). This allows IRP1 to increase expression of the transferrin receptor by stabilization of the corresponding mRNA. Simultaneously, it represses translation from ferritin mRNA ensuring that iron taken in by the cell is available for use. IRP2 is regulated by iron-dependent ubiquitination and proteasomal degradation (Salahudeen *et al.* 2009; Vashisht *et al.* 2009). In the bloodstream, iron levels are controlled principally by macrophages. Macrophages recycle iron during phagocytosis and breakdown of senescent erythrocytes. The plasma membrane of macrophages (and duodenal enterocytes) contains the protein ferroportin. Ferroportin exports Fe^{2+} from the macrophage cytoplasm into the plasma. Ceruloplasmin then oxidizes the Fe^{2+} to Fe^{3+}, thus ensuring that the concentration gradient for Fe^{2+} is maintained in the plasma direction, and that recycled iron can be taken up by circulating transferrin. Systemic iron metabolism in mammals is controlled by the peptide hormone hepcidin (see below).

Iron transport in trypanosomatids

Trypanosoma brucei. *T. brucei* differs from other human pathogenic trypanosomatids in that its lifestyle is exclusively extracellular, living as a trypomastigote in the bloodstream and tissue fluids of the mammalian host. As such, its major source of iron is transferrin (Schell *et al.* 1991*a*). *T. brucei* expresses a unique transferrin receptor which is encoded by two of the variant surface glycoprotein (VSG) expression-site associated genes (ESAGs) (Fig. 1a). Proteins encoded by *ESAG6* and *ESAG7* form a heterodimeric receptor that is attached to the membrane by a glycosylphosphatidylinositol (GPI) lipid anchor (Schell *et al.* 1991*b*; Ligtenberg *et al.* 1994; Salmon *et al.* 1994; Steverding *et al.* 1994). These two proteins appear to be adaptations of the VSG, which have evolved to bind host transferrin (Salmon *et al.* 1997). Their subcellular location is restricted to the flagellar pocket, the only part of the trypanosome surface that has endocytic activity. The ESAG6/7 transferrin receptor is expressed exclusively in the mammalian stages of the life cycle. When it is expressed ectopically in the insect stage, its distribution changes to include the whole of the plasma membrane. This implies that another factor is required to maintain flagellar pocket localization (Ligtenberg *et al.* 1994).

Each bloodstream-form expression site (ES) contains copies of *ESAG6* and 7, which have a short hypervariable region. It has been proposed by P. Borst that this could reflect variability in the affinity of the different receptors for transferrins from different mammalian hosts (Bitter *et al.* 1998; van Luenen *et al.* 2005). In light of this hypothesis, it is notable that the closely related parasite *Trypanosoma equiperdum*, which has a more restricted host range, exhibits much less diversity in its *ESAG6* genes than *T. brucei* (Isobe *et al.* 2003; Witola *et al.* 2005). Strong evidence in favour of the host-range hypothesis comes from recent studies showing the extent to which trypanosomes will go to ensure their transferrin supply (van Luenen *et al.* 2005). When trypanosomes expressing the transferrin receptor from the 221 ES are removed from bovine serum and grown in canine serum, they experience a cessation in growth after 2 days as their iron level is depleted. Within a week, growth resumes at the normal rate. Many of the outgrowing trypanosomes have switched to a new VSG expression site with different copies of the *ESAG*s; however, a number retain expression from the 221 locus. These cells are referred to as adaptors and have undergone different changes, all of which result in alterations in the expression of ESAG6 and/or 7 (Fig. 1b) In the

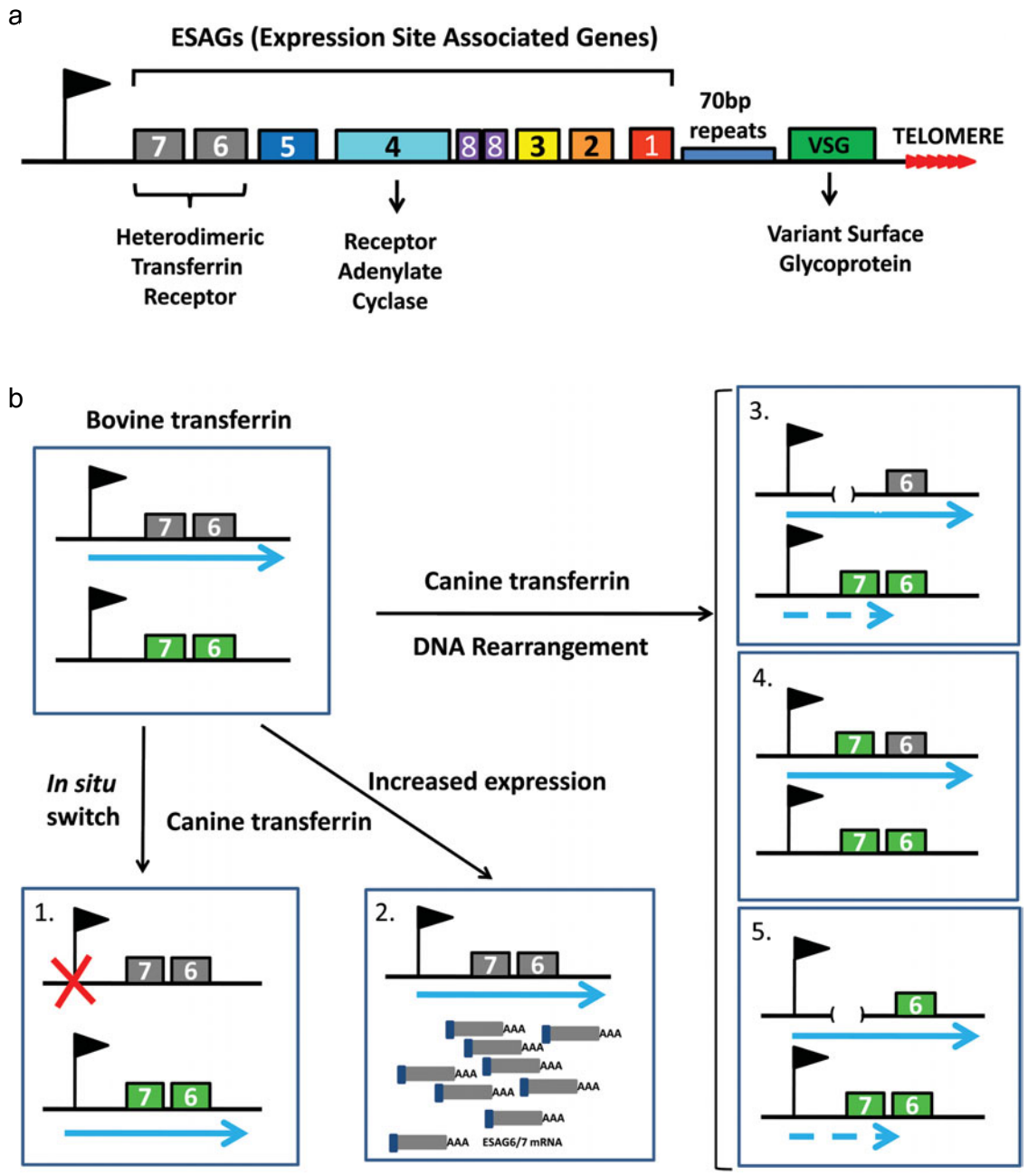

Fig. 1 (a) Map of the bloodstream-form variant surface glycoprotein expression site in *Trypanosoma brucei*. The expression-site associated genes (*ESAGs*) are illustrated along with their known functions. The promoter is indicated by the black flag. The *ESAG6* and *7* genes encoding the transferrin receptor are located proximal to the promoter, whilst the VSG gene is always the most distal. (b) Mechanisms for changing transferrin receptor expression in adapted *T. brucei*. The transferrin receptor encoded by the *ESAG6*/*7* pair from the 221 expression site (ES) (grey boxes 7 and 6) is expressed in medium containing bovine transferrin. Each silent ES contains variant copies (green boxes). The transcribed ES is indicated by the blue arrow. When trypanosomes are switched to canine transferrin, they can adapt in several ways. (1) *In situ* transcriptional switch. The old expression site is silenced (red X) and a new one activated, resulting in the expression of new *ESAG6*/*7* variants. (2) Post-transcriptional upregulation of the previously expressed *ESAG6*/*7* to increase the amount of surface transferrin receptor by increasing *ESAG6*/*7* mRNA levels. (3) Deletion (indicated by brackets) of the expressed *ESAG7* gene and transcription of a previously silent *ESAG7* (dotted blue arrow). The new ESAG7 protein forms a heterodimer with the previously expressed ESAG6. (4) Gene conversion of the existing *ESAG7* to replace it with a new *ESAG7*, again resulting in a different heterodimeric receptor. (5) Deletion of the expressed *ESAG7* coupled with gene conversion of the expressed *ESAG6*. In this case both *ESAG*s are different to the previously expressed pair, but are transcribed from different ESs. These mechanisms are based on those described by van Luenen *et al*. (2005). For simplicity only 2 expression sites are illustrated, there may be up to 20 in a single trypanosome.

simplest case, the levels of ESAG6 and 7 proteins are highly upregulated, so even with low affinity transferrin binding, the cell's iron needs are supplied (Fig. 1b, panel 2). In other cases, there is DNA rearrangement at the *ESAG6/7* locus of the 221 ES. In some adaptors, *ESAG7* is deleted from the ES and transcribed from a 'silent' ES elsewhere, the new heterodimeric receptor having a higher affinity for dog serum (Fig. 1b, panel 3). On some occasions, the *ESAG7* gene in the transcribed ES is replaced by a different copy from another ES, again creating a novel heterodimeric receptor (Fig. 1b, panel 4). Some cells do both, deleting *ESAG7* and replacing the copy of *ESAG6* with a different one from another ES (Fig. 1b, panel 5). In all 3 cases, the receptor expressed by the adaptors is different from that expressed by the parental strain. These changes only occur if no calf serum is added to the medium indicating that selection is a consequence of the need to take up enough canine transferrin.

Some investigators have questioned the relevance of host transferrin to ESAG variability (Salmon *et al*. 2005). Others have suggested that the difference in affinity only comes into play once the host has started to make significant antibody responses to the ESAG6/7 heterodimer (Steverding, 2003, 2006). Specific antibodies may compete with transferrin for the receptor and so a high-affinity receptor is required to maintain the intracellular iron levels required for trypanosome replication. A trypanosome has been estimated to take up $\sim$85 000 Fe^{3+} ions per generation but only requires $\sim$40 000, and it is therefore assumed that only 50% of the receptors need be occupied at any given time (Steverding, 1998). Calculations based on the relative K_ds of the various receptor dimers for different transferrins appear to support the hypothesis that switching expression sites is only of benefit once the anti-ESAG6/7 antibody levels rise. However, most studies on transferrin uptake in *T. brucei* have not taken into account that in chronic trypanosomiasis, especially in cattle, there is profound anaemia (d'Ieteren *et al*. 1998; Naessens, 2006) in which host transferrin levels, particularly holotransferrin, are decreased. In such a situation, antibodies against the ESAG6/7 transferrin receptor may have a far more significant effect on trypanosomal iron uptake since they are competing with decreased levels of transferrin, particularly if the expressed transferrin receptor has a low affinity for the transferrin of that host (Stijlemans *et al*. 2008). It is possible that this chronic and progressive host anaemia is one reason why bloodstream-form *T. brucei* have evolved to require relatively little iron (Steverding, 1998). The finding that strong selection for changes in ESAG6/7 expression does occur under differential serum conditions is strong evidence for a selective role of host transferrin (Mussmann *et al*. 2003, 2004; van Luenen *et al*. 2005), but does not rule out the contribution that the other ESAGs may also play in active ES selection in a given host.

The trypanosomal transferrin receptor can also be upregulated when parasites are depleted of iron using chelators (Fast *et al*. 1999). This upregulation occurs post-transcriptionally, but is not dependent on the iron regulatory activity of aconitase, in contrast to human transferrin receptor expression (see above), since upregulation still occurs in a cytosolic aconitase null mutant (Fast *et al*. 1999). The rapid upregulation of ESAG6/7 transferrin receptor expression can also be induced by moving trypanosomes expressing the transferrin receptor from the 221 ES from bovine to dog serum. Since the transferrin receptor encoded in the 221 ES has poor binding capacity for dog transferrin, this process mimics iron starvation (Mussmann *et al*. 2004), providing strong evidence that transferrin receptor expression can be profoundly influenced qualitatively (sequence) and quantitatively (expression level) by the host in which the trypanosome finds itself, and by the iron status of that host. This upregulation of ESAG6/7 expression results in mislocalization of some of the protein onto the cell surface, suggesting that the factors retaining the protein in the flagellar pocket are saturable. Cell-surface localized transferrin receptor is non-functional and is likely to consist of ESAG6 homodimers (Schwartz *et al*. 2005). An alternative explanation for apparent retention in the flagellar pocket has proposed that protein dimers with 2 GPI anchors (e.g. VSG) are targeted to the cell surface while those with 1 GPI anchor (e.g. ESAG6/7) are targeted to the flagellar pocket and cycled through the endosomal system. They are ultimately turned over by lysosomal degradation or shedding (Schwartz *et al*. 2005; Schwartz and Bangs, 2007).

After transferrin is bound to the ESAG6/7 transferrin receptor, it is endocytosed (Fig. 2), a process that requires the small GTPase TbRab5A and clathrin (Morgan *et al*. 2001; Pal *et al*. 2003). RNA-mediated knockdown suggests that the phosphatidylinositol-3 kinase TbVPS34 also plays a role in transferrin trafficking, possibly downstream of TbRab5A (Hall *et al*. 2006). The endosome acidifies and the iron is released from the transferrin, to be used by the parasite. Unlike the mammalian transferrin receptor, which remains tightly bound to its cargo throughout, ESAG6/7 loses affinity for apotransferrin at low pH. The apotransferrin is then degraded by the cathepsin B-like protein, TbcatB in the lysosome (Maier and Steverding, 1996; O'Brien *et al*. 2008), while the receptor is recycled back to the flagellar pocket via TbRab11 positive vesicles (Steverding *et al*. 1995; Jeffries *et al*. 2001). The degraded transferrin fragments are recycled to the surface and exocytosed, also by a Rab11-dependent mechanism (Pal *et al*. 2003; Hall *et al*. 2005). How the iron gets from the endolysosomal system to the

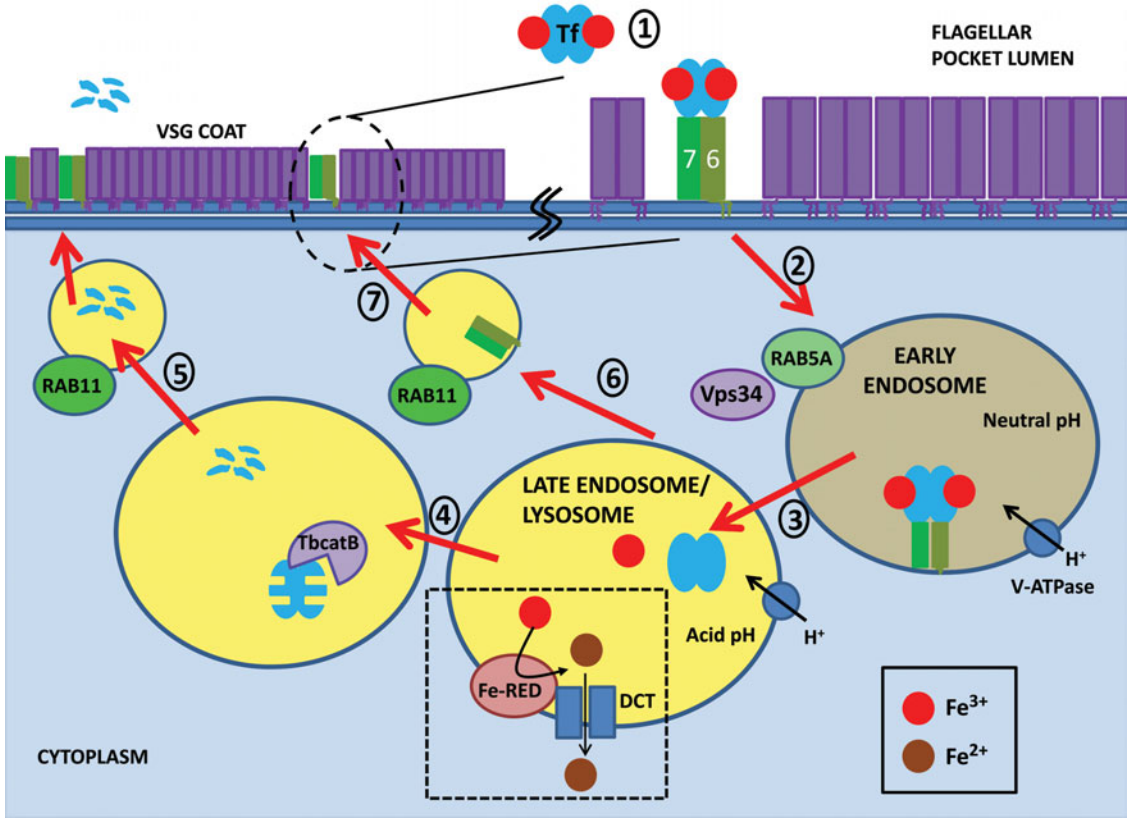

Fig. 2. The pathway for iron uptake in bloodstream-form *Trypanosoma brucei*. (1) Transferrin (Tf) binds to the ESAG6/7 heterodimer in the flagellar pocket. (2) The receptor-Tf complex is taken up into a sorting endosome under control of the small GTPase Rab 5A. The exact role of Vps34 remains to be clarified but it is required for trafficking of transferrin. (3) The endosome is acidified by the vacuolar ATPase and acquires hydrolytic enzymes. Iron is released as the pH drops and is enzymatically reduced to Fe^{2+} by a ferric reductase (Fe-RED). The Fe^{2+} is transported into the cytoplasm, via a divalent cation transporter (DCT). (4) The Tf is degraded by the protease TbcatB. (5) The Tf fragments are released into the bloodstream in a Rab11-dependent process. (6) The receptor is recycled back to the surface via Rab11 positive recycling endosomes and, (7) released onto the flagellar pocket membrane. The mechanism of iron reduction and transport into the cytoplasm have not yet been characterized and are shown in the dotted box for this reason.

cytoplasm has not yet been resolved. By analogy with other systems, this is likely to involve a ferric reductase/divalent metal ion transporter couple (dotted area, Fig. 2). The *T. brucei* genome contains 2 putative ferric reductases, a cytochrome b_{561}-type (Tb927.6.3320) and an NADPH-dependent flavoprotein (Tb11.02.1990), and either or both could fulfil this role. There are also multiple putative divalent cation transporters (e.g. Tb11.01.0725 and Tb11.01.0760), although none has yet been implicated in iron transport. The intracellular transport and metabolism of iron in bloodstream-form African trypanosomes is an area that requires further investigation.

In procyclic-form trypanosomes, rather more is known about the fate of intracellular iron, but less about its uptake. The rate of endocytosis in procyclics is greatly reduced compared to that of bloodstream-forms, even though procyclics require a greater amount of iron (Morgan *et al.* 2001). A major difference between the two stages is the metabolic activation of the mitochondrion in the procyclic stage. The change from bloodstream to procyclic-forms involves the production of an active respiratory chain terminating in a standard cyanide-sensitive cytochrome *c* oxidase. This is accompanied by the expression of TCA cycle enzymes including fumarase. These biochemical changes involve the production of FeS cluster proteins such as the Rieske protein of cytochrome *c* reductase (complex III). FeS cluster proteins are enzymes in which iron atoms are co-ordinated with inorganic sulphur, in addition to cysteine thiol groups. Two of the major players in FeS cluster assembly have been characterized in *T. brucei*. These are the cysteine desulphurase TbIscS2, and the metallochaperone, TbIscU. The first is required to remove sulphur from cysteine for incorporation into FeS clusters, and the second provides a surface on which the FeS clusters are assembled, prior to incorporation into their apoprotein. RNAi-mediated depletion of either protein is detrimental to procyclic trypanosomes and they are required for both mitochondrial and cytosolic FeS cluster protein production (Smid *et al.*

2006). As TbIscS2 and TbIscU are restricted to the mitochondrion, this suggests that all trypanosome FeS clusters are assembled in the mitochondrion and exported to the cytosol as required. 'Knockdown' of FeS cluster assembly also causes a switch in the metabolism of the mitochondrion toward a more bloodstream-like form, with increased utilization of the alternative oxidase instead of the respiratory chain, and decreased mitochondrial production of ATP and acetate. These changes are also observed when RNAi is used to deplete components of the respiratory chain, including the Rieske protein (Horvath *et al*. 2005). The switch to a more bloodstream-like mitochondrion provides a clear link between the iron-utilization machinery and life-cycle stage metabolic transitions in *T. brucei*.

TbIscS2 and TbIscU provide the sulphur and the scaffold respectively for FeS cluster assembly. The iron is donated by the highly conserved mitochondrial protein frataxin. *T. brucei* contains a frataxin homologue (Tb927.3.1000), depletion of which leads to a rapid decrease in the activities of several FeS-dependent enzymes including aconitase, fumarase and succinate dehydrogenase, while the activity of non FeS-dependent proteins remains unaffected (Long *et al*. 2008*b*). In contrast to other systems, RNAi-mediated knockdown of frataxin is not accompanied by an increase in free iron, suggesting that trypanosomes may have the facility to store or remove iron before it builds up to deleterious levels. There is, however, an increase in reactive oxygen species in frataxin-depleted cells (Long *et al*. 2008*b*). Aspects of frataxin function appear to be well-conserved across a billion years of evolution, as the human frataxin gene can rescue the *T. brucei* RNAi cell line, and the trypanosomes also process the human protein in the same way as human cells. Interestingly, mutation of the signal-sequence cleavage site to abrogate processing indicates that the human frataxin does not require cleavage to carry out its function in a trypanosome mitochondrion. This contrasts with the situation in human mitochondria, where processing is necessary (Condo *et al*. 2007; Long *et al*. 2008*a*). *T. brucei* remains the only trypanosomatid in which FeS cluster biogenesis has been characterized.

Leishmania spp. Iron uptake has been relatively little studied in most species of *Leishmania*. The exceptions are *L. amazonensis* and *L. chagasi*/*infantum*[1], although the whole pathway has not been elucidated in either. In the mammalian host, *Leishmania* reside in an acidified phagolysosomal compartment in macrophages. In this compartment, they can take iron directly from host-endocytosed transferrin, without the need for their own transferrin endocytosis system, since the iron is released in the low pH milieu. All they need is a ferric reductase and a divalent cation transporter (Fig. 3). Two such activities have been identified (see below).

Binding of transferrin and lactoferrin by promastigotes of *L. chagasi* has been shown, although the receptors responsible have not been characterized at the gene level (Wilson *et al*. 1994, 2002). A 70 kDa protein binds both, in a non-specific manner (Voyiatzaki and Soteriadou, 1992; Wilson *et al*. 2002) but, as mentioned above, a transferrin receptor is not required for acquisition of iron within the phagolysosome. The ferric reductase activity identified in *Leishmania* derives electrons from NADPH rather than ascorbate (Wilson *et al*. 2002). This suggests that the Cytb_{561} ferric reductase (LinJ30_V3.2050) is not involved since this protein typically uses ascorbate as an electron donor. Instead, it is likely to be the flavoprotein reductase encoded by LinJ30_V3.1630, which uses NADPH. In *L. amazonensis*, *Leishmania* Iron Transporter 1 (LIT1), a member of the ZIP (Zrt/IRT-like proteins) divalent cation transporter family has been characterized and shown to be an iron transporter (Huynh *et al*. 2006). This protein is localized to the plasma membrane reflecting that *Leishmania* amastigotes can obtain iron directly from the lumen of the phagolysosome, unlike the extracellular *T. brucei* (Fig. 3). As with other members of the ZIP family, LIT1 has 8 predicted transmembrane domains. This transporter is required for intracellular replication and correct parasitophorous vacuole formation, as demonstrated by LIT1 null mutants (Huynh *et al*. 2006). Phagocytosed LIT1 null mutants differentiate into intracellular amastigotes as normal, and vacuole maturation is not inhibited since the vacuolar membranes still acquire the lyososmal marker Lamp1. However, the development of parasites in the vacuole is severely compromised. In *L. amazonensis* infections, the parasitophorous vacuole expands as the amastigotes replicate, but in the LIT1 mutants the vacuole remains small and there is no replication of amastigotes. Instead, they appear to degenerate within the vacuole. This inability to multiply within the host was also apparent *in vivo* as there is no lesion development in infected mice, although parasites persist within the skin (Huynh *et al*. 2006).

It has been demonstrated that *L. amazonensis* reroute transferring bearing endosomes to fuse with the parasitophorous vacuole, a modified phagosome, thus ensuring a constant supply of iron to replicating amastigotes (Borges *et al*. 1998). The mechanism of interference with the host endocytic pathway has not been elucidated. There is also evidence that

[1] *L. chagasi* was considered a separate species, but it is now known to belong to the *L. infantum* clade, probably brought to Latin America by European immigration/colonization. **Mauricio, I. L., Stothard, J. R. and Miles, M. A**. (2000). The strange case of *Leishmania chagasi*. *Parasitology Today*, **16**, 188–189. The name *L. chagasi* is used here as this was the designation used in the cited work.

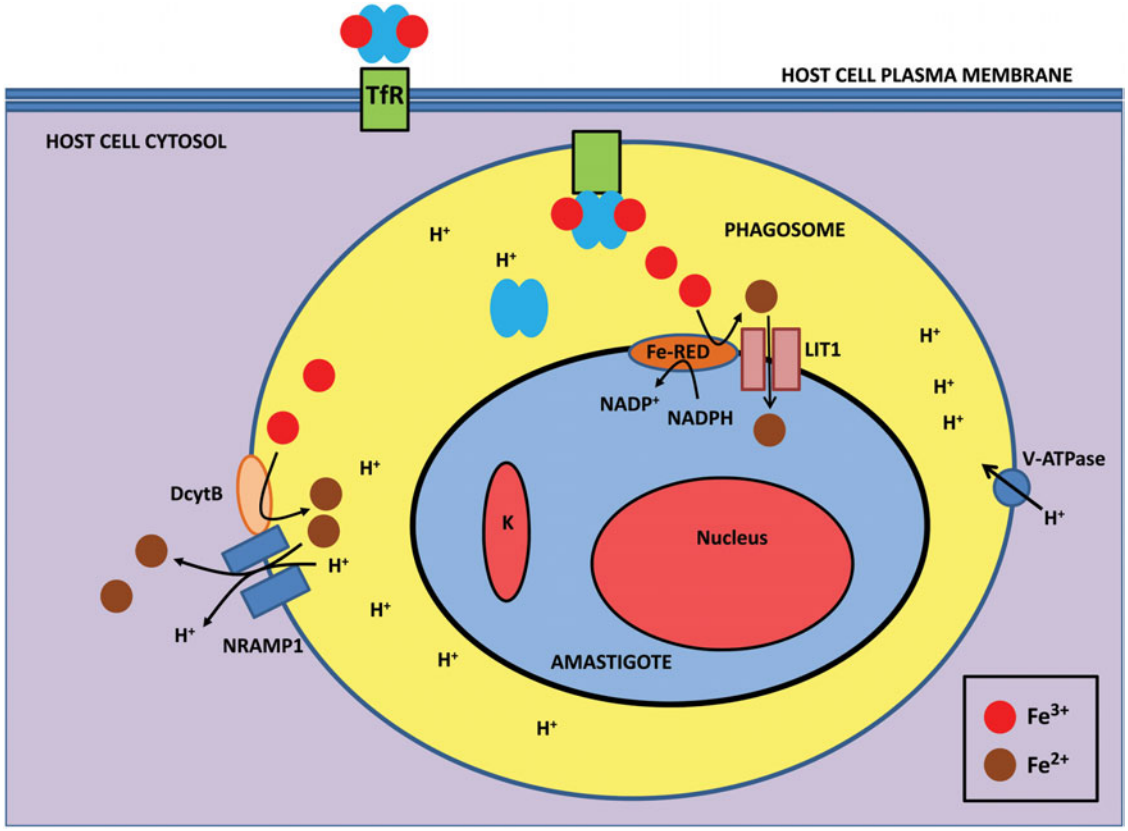

Fig. 3. Iron uptake in *Leishmania* amastigotes. Transferrin is taken up by the infected mammalian cell via its transferrin receptor (TfR). The Tf:TfR complex is endocytosed and the endosome fuses with the phagosome. Fe^{3+} is liberated from the transferrin due to the low pH. A parasite cell surface NADPH-dependent ferric reductase reduces Fe^{3+} to Fe^{2+}. The Fe^{2+} is then transported into the parasite by the divalent ion transporter LIT1. The amastigote may also acquire iron from the cytoplasmic labile iron pool by an undefined mechanism. Simultaneously, the infected cell tries to deplete iron from the phagosome through the concerted action of the Dcytb ferric reductase and the NRAMP1 divalent cation transporter. K: kinetoplast.

Leishmania may act to increase the uptake of holotransferrin by the host cell. Infection with *L. donovani* was shown to stabilize transferrin receptor mRNA and to promote uptake of radio-labelled iron from holotransferrin. *L. donovani* depletes the intracellular labile iron pool leading to the activation of iron regulatory proteins, which then decrease ferritin expression and upregulate transferrin receptor 1 (Das *et al*. 2009).

Leishmania also require a source of porphyrins since they lack the biosynthetic capacity for their production. Recent work has identified haemoglobin as a source of iron for intracellular amastigotes (Carvalho *et al*. 2009). *L. infantum* axenic amastigotes were unable to grow on iron-depleted medium, but growth could be restored by addition of haemoglobin. A 46 kDa flagellar pocket protein of *L. donovani* had previously been identified as the haemoglobin receptor (Krishnamurthy *et al*. 2005). Carvalho *et al*. (2009) showed that antibodies against this receptor could block the utilization of haemoglobin. The exploitation of haem as an iron source by amastigotes may explain why the LIT1 ferrous iron transporter null mutants were able to persist in infected mice (Huynh *et al*. 2006). In this respect, it is notable that the major replication sites of visceralising *Leishmania* species are also sites of macrophage erythrocyte recycling. Erythrocyte haemoglobin could therefore provide intracellular amastigotes with a ready source of haem iron. *Leishmania*, unlike the trypanosomes, possess an orthologue of ferrochelatase (LmjF17.1460), which is functional, since protoporphyrin IX can replace haemin in culture medium for *L. amazonensis* (Chang and Chang, 1985).

It is clear that *Leishmania* are capable of obtaining iron from a number of host sources. The presence of the iron transport machinery on the surface of the *Leishmania* amastigote, together with the demonstration that genetic disruption of iron uptake completely abrogates infection, implicates this machinery as an Achilles' heel of the parasite suitable for targeting with chemotherapy.

Trypanosoma cruzi. Almost nothing is known about iron uptake or iron sources in *T. cruzi*. Given the wide range of hosts and host cells infected, it is possible that *T. cruzi* utilizes several different iron sources, such as myoglobin in cardiac muscle cells – a source of both iron and porphyrins. The

cytoplasmic habitat of *T. cruzi* also gives it access to the host's iron storage protein, ferritin. This could be particularly relevant to amastigotes in hepcidin-responsive macrophages (see below). Insect-stage epimastigotes have a requirement for both haem and non-haem iron *in vitro* (Lalonde and Holbein, 1984).

Transferrin binds to and is taken up by *T. cruzi* amastigotes *in vitro* (Lima and Villalta, 1990). However, the physiological relevance of this interaction is unclear since amastigotes replicate in the cytoplasm of host cells, a niche in which transferrin is conspicuously absent. Consistent with this, very little intracellular staining has been detected in amastigotes using gold-labelled transferrin (Soares and de Souza, 1991). This does not exclude the possibility that trypomastigotes could pick up transferrin in the bloodstream, or that epimastigotes can acquire iron from transferrin in the bloodmeal. Both life-cycle stages have been shown to bind transferrin, and in epimastigotes it accumulates in the reservosome, a late endosome/lysosome–like organelle (Soares and de Souza, 1991; Soares *et al.* 1992). Despite these preliminary studies, the uptake and usage of iron by *T. cruzi*, particularly the intracellular amastigote, is poorly understood. Given the large number of people infected, or at danger from this parasite, and the dearth of drugs available, a deeper understanding of parasite iron requirements could be fundamental to new chemotherapeutic strategies.

How do trypanosomatids utilize scavenged haem: questions to be answered

One problem that arises from the use of haem by all the pathogenic trypanosomatids is how the haem ring is broken to release iron (Chang and Chang, 1985; Lara *et al.* 2007). There is no readily identifiable orthologue of haem oxygenase in the genome sequences of any of the trypanosomatids, although a putative activity has been measured in promastigotes of *L. donovani* (Srivastava *et al.* 1997). Additionally, trypanosomes also lack ferrochelatase (present in *Leishmania*), and so, theoretically, cannot insert iron into scavenged porphyrins. This raises the possibility that trypanosomatids accomplish breakage of the porphyrin ring and insertion of iron into protoporphyrin by a different mechanism from other organisms. However, the simplest explanation for the use of haem, given the apparent lack of both haem oxygenase and ferrochelatase, is that trypanosomes simply incorporate scavenged haem directly into apoproteins without going through intermediate steps. This would then require that the iron used by non-haem proteins be obtained independently from mammalian or insect sources. It is implicit from this that one source of iron would not be able to complement the loss of the other.

THE ROLE OF IRON IN TRYPANOSOMATIDS

Iron has multiple roles in most organisms and trypanosomatids are no exception, with many activities common to both parasite and host. These include the reduction of ribonucleotides for DNA synthesis and cytochrome-based oxidative respiration. Ribonucleotide reduction utilizes a classical eukaryotic enzyme (Dormeyer *et al.* 1997; Hofer *et al.* 1997), with the major difference in trypanosomatids being that the reducing power is provided by the kinetoplastid-specific thiol trypanothione in a reaction catalysed by tryparedoxin (Dormeyer *et al.* 2001). Below, we discuss several examples of iron-dependent processes in trypanosomatids, which are distinct from the mammalian host. These could have potential as targets for therapeutic intervention.

Antioxidant defences

Fe-dependent superoxide dismutases (*Fe-SODs*). Unlike their mammalian hosts, which contain Cu/Zn and Mn-dependent SODs, trypanosomatids express 4 different Fe-dependent superoxide dismutases (Dufernez *et al.* 2006; Wilkinson *et al.* 2006). Fe-dependent SODs are restricted to bacteria and some protozoa. These enzymes help to protect the cell from oxidative stress by catalysing the dismutation of superoxide radicals into H_2O_2 and O_2, the H_2O_2 then being metabolized by peroxidases. Iron removes an electron to oxidize 1 molecule of superoxide (step 1), and is then reoxidized, by reaction with a second superoxide molecule in the presence of protons (step 2).

1. $\text{Fe(III)-SOD} + O_2^{\bullet -} \rightarrow \text{Fe}^{2+}\text{-SOD} + O_2$

2. $\text{Fe}^{2+}\text{-SOD} + O_2^{\bullet -} + 2H^+ \rightarrow \text{Fe(III)-SOD} + H_2O_2$

The net result of these two reactions is regeneration of Fe(III)-SOD and the production of 1 molecule each of O_2 and H_2O_2 from 2 superoxide radicals (Halliwell and Gutteridge, 2007).

The 4 enzymes present in trypanosomatids are differentially compartmentalized. In *T. brucei*, SOD A and SOD C are found in the mitochondrion whilst SOD B1 and B2 are glycosomal, with some SOD B1 present in the cytoplasm. Phylogenetic analyses have indicated that the trypanosomatid Fe-SODs belong to 2 distinct clades, with SOD A and SOD C in one group and the SOD Bs in the other. Both sets of *SOD*s appear to have been acquired by lateral gene transfer from prokaryotes (Dufernez *et al.* 2006). RNAi-mediated knockdown of the Fe-SODs in *T. brucei* demonstrates that SOD B1/B2 expression is essential in bloodstream forms. However, the RNAi construct could not discriminate between the two isoforms due to their extreme nucleotide sequence conservation (Wilkinson *et al.* 2006). The ability to generate SOD B1 and SOD B2 individual

null mutants with no specific defects in growth or virulence indicates that the two isoforms can complement each other, but that at least one must be expressed for survival (Prathalingham *et al*. 2007). However, there was a difference between the two null mutants in susceptibility to trypanocidal agents. SOD B1 mutants were more susceptible to benznidazole and nifurtimox than the wild type, whereas SOD B2 mutants had comparable sensitivity (Prathalingham *et al*. 2007). This argues that one-electron reduction of the drugs takes place in the cytoplasm where SOD B1 is at a higher level, rather than the glycosome. The two mitochondrial isoforms SOD A and SOD C are dispensible for the bloodstream form; however, RNAi-mediated knockdown of SOD A renders cells more susceptible to the superoxide generator paraquat (Wilkinson *et al*. 2006). SOD A is expressed in the less metabolically active mitochondrion of the bloodstream form, probably to protect the kinetoplast DNA, and by implication, turnover of paraquat occurs in this organelle. In contrast, knockdown of SOD C produces no discernible effects, either on growth or susceptibility to oxidizing agents and the role of SOD C remains to be resolved.

In *T. cruzi*, SOD A has been implicated in the control of programmed cell death. Inducible overexpression of SOD A protects the parasite from serum-mediated death (Piacenza *et al*. 2007), demonstrating a direct role for superoxide radicals in the cell death signalling programme. TcSOD A appears to be concentrated around the kinetoplast suggesting a role in protecting mitochondrial DNA from oxidative damage (Taylor and Kelly, 2006). A similar location has been noted for the mitochondrial peroxiredoxin (Wilkinson *et al*. 2000). It is tempting to speculate that both enzymes, together with others, constitute an antioxidant complex guarding kDNA from oxidative damage.

Increased expression of the *T. cruzi* SOD B1 isoform results in greater susceptibility to the superoxide generator gentian violet (Temperton *et al*. 1998). This seemingly paradoxical outcome has also been observed in bacteria and mammalian cells, where SOD activity has been upregulated. A possible explanation is that higher SOD activity results in increased H_2O_2 levels. In situations where the ability to metabolize H_2O_2 is limited, this can result in the production of $OH^{\bullet}$ radicals by the Fenton reaction. Of more interest, the SOD B1 overexpressers were also more susceptible to the trypanocidal drug benznidazole, but not to the nitrofuran drugs, in contrast to *T. brucei* (Temperton *et al*. 1998). This suggests that at least part of the trypanocidal mechanism of benznidazole is mediated through increased oxidative stress in the cytoplasm or glycosome. The glycosomal SODs B1 and B2 are developmentally regulated in *Leishmania*, with SOD B1 being more highly expressed in amastigotes and SOD B2 in promastigotes (Plewes *et al*. 2003). A single allele deletion of SOD B1 was found to confer a lower survival rate in macrophages or when exposed to paraquat, suggesting that glycosomal SOD activity is important in *Leishmania* amastigotes (Plewes *et al*. 2003).

It is clear that the Fe-dependent SODs, particularly the glycosomal isoforms, play important roles in pathogenic trypanosomatids and that they or their iron co-factor are suitable targets for drug development. Inhibitors of SOD could then be used in combination with other trypanocidal drugs, such as benznidazole, to potentiate their effects, thus minimising the effective dose, and decreasing toxic side effects.

Ascorbate-dependent peroxidase

Trypanosomatids lack the classical catalase and selenium-dependent glutathione peroxidases which can rapidly metabolize H_2O_2. Instead, they express a battery of different peroxidases including tryparedoxin-dependent peroxiredoxins, and non-selenium dependent glutathione peroxidases (Flohe *et al*. 1999; Irigoin *et al*. 2008; Wilkinson and Kelly, 2003). In addition, *T. cruzi* and *Leishmania* possess an ascorbate-dependent peroxidase (APX), which can metabolize hydrogen peroxide but not organic peroxides (Wilkinson *et al*. 2002*a*; Adak and Datta, 2005). Ascorbate peroxidases are more usually plant enzymes and different isoforms are specific to different compartments of the plant cell, including both stromal and thylakoid compartments of the chloroplast. APX is a haemoprotein belonging to the catalase-peroxidase superfamily, and is absent from African trypanosomes.

The catalytic mechanism of APX includes 3 steps. The first involves oxidation of the haem iron to the ferryl (Fe(IV)) state by the H_2O_2 molecule to form compound I (underlined below), coupled with the formation of a cationic radical on the porphyrin ring. Compound I therefore has an oxidation state of +5.

1. $Fe3^{+}$-porphyrin $+ H_2O_2 \rightarrow \underline{Fe4^{+}{=}O\ porphyrin^{\bullet +}} + H_2O$

Compound I then reacts sequentially with 2 molecules of ascorbate (Asc) to produce the ascorbyl free radical (AFR, monodehydroascorbate) and regenerate the resting enzyme (oxidation state +3).

2. $Fe4^{+}{=}O\ porphyrin^{\bullet +} + Asc \rightarrow Fe4^{+}{=}O + AFR^{\bullet}$

3. $Fe4^{+}{=}O + Asc \rightarrow Fe3^{+}$-porphyrin $+ AFR^{\bullet}$

The net reaction is: $H_2O_2 + 2\ Asc \rightarrow H_2O + 2\ AFR^{\bullet}$

Ascorbate is only required to return the enzyme to its resting state. In the absence of ascorbate,

the porphyrin π-cation radical transfers to cysteine or tryptophan residues in the protein chain and the enzyme can become irreversibly oxidized (Hiner *et al*. 2001; Kitajima *et al*. 2008). The AFR is reduced back to Asc by the action of a number of small molecule reductants (e.g. trypanothione, (Krauth-Siegel and Ludemann, 1996)) or redox active enzymes. It is not clear whether any other reductant can fulfil the role of ascorbate in the above reaction.

Although *Leishmania* and *T. cruzi* proteins are clearly derived from the same ancestral gene (62% amino acid identity between *L. major* and *T. cruzi* APXs), they now appear to have different biological roles. The *Leishmania* enzyme is mitochondrial and has a classical mitochondrial targeting sequence, whereas the *T. cruzi* protein has a much longer N-terminal extension, which seems to be related to plastid transit peptides, and is resident in the endoplasmic reticulum (Wilkinson *et al*. 2002*a*). Why the proteins should be differentially targeted in 2 related organisms is not obvious. *Leishmania* APX appears to have a role in protection of mitochondrial membrane lipids from oxidative stress and its expression is induced on exposure to H_2O_2 (Dolai *et al*. 2008). In the ER, H_2O_2 is a constitutive by-product of the Ero1/protein disulphide isomerase redox cycle and it is likely that the *T. cruzi* APX plays an analogous role to *Leishmania* APX, protecting membrane lipids in the ER membrane from peroxidation. *T. cruzi* also expresses another peroxidase in the ER, glutathione-dependent peroxidase II (GPX II), which cannot metabolize H_2O_2, but does metabolize phospholipid hydroperoxides (Wilkinson *et al*. 2002*b*). Thus, it is likely that these two proteins act in concert to protect the ER membrane from the consequences of oxidative stress.

Trypanosome alternative oxidase (*TAO*)

Bloodstream-form African trypanosomes are dependent on glycolysis since the normal mitochondrial respiratory chain is absent. Instead, oxidation of ubiquinol is carried out by the so-called alternative oxidase (TAO), resulting in the net transfer of electrons from glycerol-3-phosphate to molecular oxygen to produce water. Unlike the conventional respiratory chain, the transfer of electrons to TAO does not result in the generation of ATP and it does not create a proton gradient (Clarkson *et al*. 1989). TAO is not present in the mammalian host (or in *T. cruzi* and *Leishmania*, although there is a second TAO-like sequence in all trypanosomatids examined (Chaudhuri *et al*. 2006)). In contrast to the other respiratory oxidases, TAO is composed of a single protein of 37·5 kDa and has 2 iron-binding sites constituted by conserved glutamate and histidine residues on helices 1,3,4 and 5 (Chaudhuri *et al*. 2006). The current model for the alternative oxidase active site suggests that TAO is a member of the diiron carboxylate protein family (Andersson and Nordlund, 1999). These proteins are characterized by the motif E-X_n-E-X-X-H-X_n-E-X_n-E-X-X-H, where E is glutamate and H is histidine, with X being any amino acid; n signifies a number of residues. The 4 carboxylates of the glutamates and imidazole nitrogens of the histidine co-ordinate 2 iron atoms in the catalytic centre, hence the name diiron carboxylate protein (Berthold and Stenmark, 2003). Although the *T. brucei* TAO, like the other alternative oxidases, has 2 strongly hydrophobic segments, it is thought to be positioned along the matrix face of the inner mitochondrial membrane rather than spanning the membrane (Andersson and Nordlund, 1999; Chaudhuri *et al*. 2006). Site-directed mutagenesis of the putative iron-binding ligands H165, E214, E266 and H269 in TAO has demonstrated their importance to activity. Any one of these mutations abolishes the ability of the TAO gene to complement an *E. coli* haem-deficient mutant (Ajayi *et al*. 2002). Iron-dependence of TAO has also been established by iron chelation with *o*-phenanthroline, resulting in strong inhibition which was reversed by addition of iron, but not other metals such as copper (Ajayi *et al*. 2002).

In *T. brucei*, TAO is developmentally regulated with the mRNA level dropping rapidly on differentiation from bloodstream to procyclic-forms (Chaudhuri *et al*. 2002). This differential mRNA accumulation appears to be due to a change in the half-life of the mRNA. This is mediated by a labile protein factor since the transcript is stabilized by cycloheximide treatment. During the transition to metacyclic trypomastigotes, which are pre-adapted for infection of the mammalian host, TAO activity is again upregulated and the standard cyanide-sensitive respiratory chain downregulated (Bienen *et al*. 1991). Although the level of TAO expressed in procyclics is lower than in bloodstream-forms, it also plays an important role in this stage, as inhibition of the cyanide sensitive respiratory chain is not lethal unless the TAO is inhibited simultaneously (Coustou *et al*. 2003).

Due to the reliance of bloodstream-form African trypanosomes on glycolysis, coupled to the glycerol-3-phosphate oxidation required to regenerate NAD^+, TAO has been proposed to be a candidate drug target (Yabu *et al*. 2003; Chaudhuri *et al*. 2006). However, computer modelling of metabolic flux and RNAi-mediated depletion of TAO suggests, *in vitro*, that the TAO protein would have to be inhibited by more than 95% for there to be a significant effect on trypanosome growth (Helfert *et al*. 2001). In *T. brucei*, TAO inhibition is only lethal when coupled with administration of glycerol to block glycerol-phosphate dehydrogenase or with repeated administration of the inhibitor (Yabu *et al*. 1998,

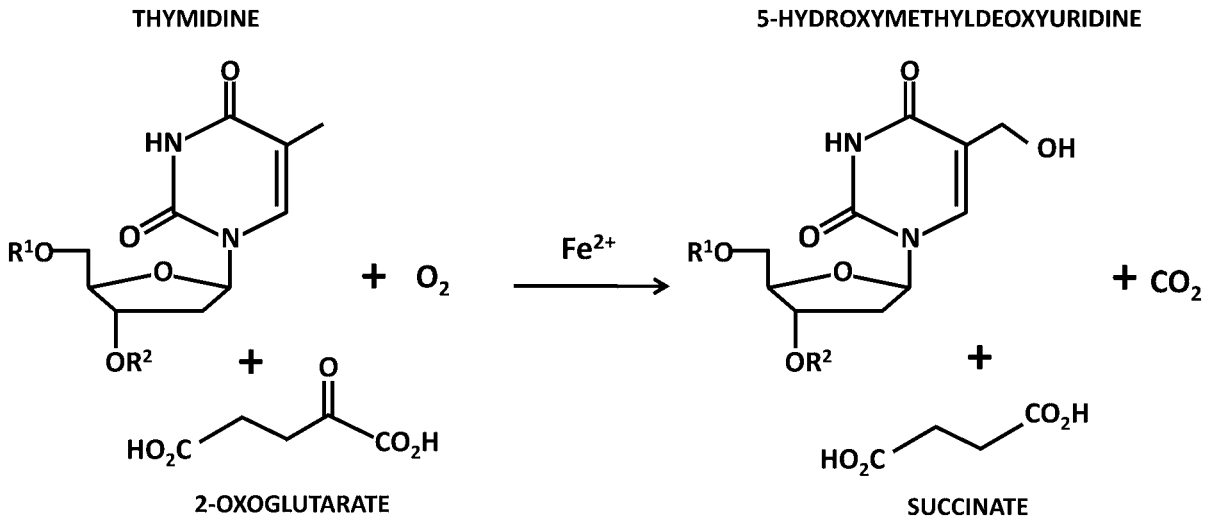

Fig. 4. Hydroxylation of thymidine residues in DNA. Thymidine hydroxylases JBP1 and JBP2 use oxidative decarboxylation of 2-oxoglutarate in the presence of molecular oxygen to add a hydroxyl group to the methyl carbon of the thymidine base. The reaction is catalysed by Fe^{2+}. R^1 and R^2 represent the 5′ and 3′ends of this strand of the DNA duplex.

2003). Nevertheless, the demonstration that inhibitor treatment can cure mice suggests that the TAO may be a viable drug target. In *T. vivax*-infected mice, a single dose of ascofuranone (50 mg kg^{-1}) has been shown to elicit cure without glycerol (Yabu *et al*. 2006).

J-base biosynthesis

A unique feature of trypanosomatids is the presence of a hypermodified base within their DNA. This base, β-D-hydroxymethyldeoxyuridine, more usually known as J, is a derivative of thymidine and is synthesized within the DNA strand in a two-step modification process. The first step in J-base synthesis involves the hydroxylation of thymidine residues in DNA to provide a hydroxyl group to which the glucose moiety is attached (Fig. 4). The hydroxylase which carries this out, is a member of the Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases, although the similarity to other members of this family is not immediately obvious (Yu *et al*. 2007). These enzymes use molecular oxygen to donate the oxygen atom required for the hydroxyl group. During this process, the iron is probably oxidized to an oxyferryl (Fe(IV)=O) state as in the APX reaction, except that in this case, the oxygen atom is derived from molecular oxygen rather than H_2O_2 (Schofield and Zhang, 1999).

There are 2 thymidine hydroxylases in trypanosomatids, referred to as J-binding protein 1 (JBP1) and J-binding protein 2 (JBP2). Both contain homologous thymidine hydroxylase domains and mutation of the putative iron-binding residues in *Leishmania* JBP1 (H189, D191 and H239) renders the protein unable to rescue J-base biosynthesis in JBP1 null mutant *T. brucei*, as does mutation of the conserved arginine residue required for 2-oxoglutarate binding (R255) (Yu *et al*. 2007). These mutations do not affect the DNA binding of the enzyme and so the inference is that they affect the catalytic capability, as would be expected if they were required for iron incorporation. Similar mutagenesis studies on iron and 2-oxoglutarate binding residues, also confirm that JBP2 is a member of this family (Cliffe *et al*. 2009; Vainio *et al*. 2009). In neither case has the hydroxylase activity been reconstituted *in vitro*. Whilst J-base appears to be dispensible for *T. brucei* (and is absent in the procyclic stage), JBP1 is essential in *Leishmania* (Genest *et al*. 2005). The situation in *T. cruzi* is unclear.

Iron and anti-kinetoplastid drugs

There have been few studies directly addressing the role of iron in drug-mediated killing of kinetoplastid parasites. Indeed, for most clinically relevant anti-kinetoplastid drugs, the exact mechanism of action remains unknown and many of their effects may be pleiotropic. However, a recent study has implicated iron in drug activity (particularly metalloids) in *Leishmania* (Mehta and Shaha, 2006). Using deferoxamine (DFO), Mehta and Shaha showed that iron depletion inhibits changes in the mitochondrial membrane potential and ATPase activity produced by antimonial or arsenical drugs. This inhibition can be reversed if the DFO is saturated with iron prior to incubation with the parasites, showing that the effect is dependent on iron chelation. In addition, it has been demonstrated that DFO can reduce the level of cell death induced by treatment with SbIII or AsIII, whereas addition of iron causes a slight exacerbation of cell death. The changes in mitochondrial membrane potential and activity prior to drug-induced

death mirrors the mechanism shown in *T. cruzi* for complement-induced programmed cell death of epimastigotes, which is mediated by mitochondrial $O_2^{\bullet-}$ (Piacenza *et al*. 2007). Thus, the drug-induced death of *Leishmania* may involve activation of their cell death programme at the mitochondrial level. Exacerbation of this process by iron is likely to reflect increased oxidative stress resulting from $OH^{\bullet}$ generated by the superoxide-driven Fenton reaction.

Further studies of the role of iron in the mechanism of drug action/resistance in trypanosomatids are clearly warranted. This is particularly the case with drugs that may trigger oxidative stress, such as Ornidyl (difluoromethylornithine). Ornidyl blocks polyamine biosynthesis and therefore prevents the production of trypanothione (N^1,N^8-bisglutathionylspermidine) (Fairlamb *et al*. 1987). Almost all the antioxidant defences in trypanosomatids derive their reducing power ultimately from trypanothione. Therefore, prolonged inhibition of spermidine synthesis will result in increased oxidative stress by depletion of trypanothione, hence the level of iron available to the Fenton reaction may be key to the trypanocidal activity. As iron has also been implicated in the action of benznidazole (via SOD B1), it would seem important to dissect this interaction for future drug design (Temperton *et al*. 1998; Prathalingham *et al*. 2007; Francisco *et al*. 2008).

IRON IN PATHOGENESIS AND IMMUNITY

The peptide hormone hepcidin is released from the liver during infection. One of its roles is to prevent iron release from macrophages into the bloodstream. This is achieved by destabilization of the ferroportin molecule which is internalized and degraded, leading to a block in iron export and an increase in ferritin-bound iron in macrophages (Nemeth *et al*. 2004, 2006). In addition, hepcidin promotes degradation of ferroportin on duodenal enterocytes, thereby reducing dietary iron intake. It has also been demonstrated that macrophages can produce hepcidin themselves under the control of Toll-like receptor 4 signalling, creating an autocrine feedback loop resulting in iron sequestration (Peyssonnaux *et al*. 2006). In this way, plasma iron levels are decreased, and bloodstream pathogens prevented from accessing iron. An unfortunate effect of this innate immune strategy is to create a situation of functional anaemia in the host. This condition, known as 'anaemia of chronic infection' illustrates the capacity of iron to be both beneficial and dangerous, often simultaneously.

Trypanosoma brucei. For a bloodstream parasite such as *T. brucei*, this anaemia can create problems as it is dependent on its host for iron. In animal models, anaemia during *T. brucei* infection is type I cytokine driven and appears to be specifically targeted at removal of iron from the plasma during the chronic phase, thus reducing the pool of iron available to the circulating trypomastigotes (Stijlemans *et al*. 2008). Using C57Bl/6 mice, it is apparent that anaemia in experimental trypanosomiasis occurs in 2 distinct waves, corresponding to the acute and chronic phases of the infection. During the acute phase, there is a rapid drop in the number of erythrocytes, which then recovers, although not to uninfected levels. This recovery is followed by a continuous decrease as the chronic phase takes over. Transferrin mRNA levels increase rapidly during the acute phase, possibly in response to the removal and degradation of transferrin by the multiplying trypanosomes, but then drop back and fall below the baseline level during the chronic phase. Ferroportin mRNA levels also decrease during the chronic phase. At the same time, ferritin mRNA levels increase and remain high, as does the mRNA for divalent metal transporter 1. These findings show that the infected host responds by increasing the sequestration of iron within the macrophage cytoplasm and decreasing export into the plasma. This sequestration leads to decreased erythropoiesis as iron is unavailable for incorporation into haemoglobin. It may be this cytokine driven anaemia during chronic infection that promotes the selection of different transferrin receptors on the trypanosome. A higher affinity receptor may be required when serum holotransferrin levels drop, particularly as the trypanosome receptor cannot distinguish holo from apotransferrin.

Leishmania – the role of NRAMP1

For many years it has been established that 'natural resistance associated macrophage protein 1' (NRAMP1, now known as Slc11a1[2]) is involved in resistance to several intracellular pathogens, including *Salmonella typhimurium, Mycobacterium bovis* and *Leishmania* (Vidal *et al*. 1995). Mice strains carrying a single mutation in transmembrane helix 4 (G169D) are more susceptible to these pathogens than are strains carrying wild type *NRAMP1* alleles. Resistance can be restored by reintroducing a wild type copy of the gene, whilst NRAMP1 null mutant mice are susceptible to these pathogens (Govoni *et al*. 1996). NRAMP1 is a late endosomal/lysosomal protein expressed in macrophages and granulocytes. It functions as a divalent cation/proton symporter which transports metal ions down a proton gradient across the phagosomal membrane (Biggs *et al*. 2001) and is related to the major divalent metal ion

[2] We use the name NRAMP1, as this term is used in most papers referring to its role in infection. Slc11a1 is the systematic name: Solute carrier family 11 member 1. The Slc 11 family are proton-coupled divalent metal ion transporters.

transporter DMT1 (NRAMP2/Scl11a2). The net effect of NRAMP1 activity in acidified phagosomes is to deplete iron (and some other transition metals) from the lumen of the phagosome, thus denying pathogens that reside within this compartment access to the metal. Iron chelators can mimic the effect of NRAMP1 in null mutant macrophages infected with *Salmonella*. This suggests that NRAMP1 mediates its antimicrobial effect through its iron transport properties, although the mechanism by which this controls such diverse pathogens has yet to be established (Jabado *et al*. 2003). In *Leishmania*, the LIT1 iron transporter gene is upregulated more rapidly when parasites infect wild type cells than NRAMP1 null cells, suggesting that NRAMP1$^+$ phagosomes are depleted in iron (Huynh *et al*. 2006). In this context, it is interesting that cells infected with *L. amazonensis* mutants defective in the LIT1 iron transporter do not develop the expected parasitophorous vacuole. Since the parasites cannot transport iron from the phagosome, but still express a ferric reductase, it is probable that they enhance the natural activity of NRAMP1 by providing reduced iron at a greater rate than in cells infected with wild type *Leishmania* (Huynh *et al*. 2006). The mechanisms identified in mice may also be present in humans (Bucheton *et al*. 2003; Mohamed *et al*. 2004; Blackwell *et al*. 2009). Genetic polymorphism studies in human populations have shown a linkage between mutations in the NRAMP1 promoter region and susceptibility to visceral leishmaniasis. In the new era of genomics and deep sequencing, there is scope for much wider studies looking at the role of iron metabolism in susceptibility to VL.

Trypanosoma cruzi. Acute *T. cruzi* infection in mouse models involves anaemia which can be reversed or blocked by administration of the trypanocidal drug nifurtimox (Marcondes *et al*. 2000). In addition, anaemia is an indicator of the reactivation of Chagas disease in heart-transplant recipients (Theodoropoulos *et al*. 2009). In the mouse model, the mechanism for the anaemia was postulated to be a decrease in the lifespan of erythrocytes. However, recent studies have shown that resistance to *T. cruzi* infection involves a key inducible regulator of haematopoeisis, LRG-47, a member of the p47-GTPase family of interferon-induced proteins. These GTPases are thought to play a major role in defence against intracellular pathogens, particularly phagosomal pathogens such as *Salmonella* and *Mycobacteria* (MacMicking, 2005). They are also involved in the regulation of autophagy as an innate defence mechanism against intracellular pathogens (Gutierrez *et al*. 2004).

LRG47 null mutant mice develop severe anaemia when infected with *T. cruzi*, coupled with a general failure of haematopoiesis. These mice are susceptible to trypanosome infection, dying within 19 days, whereas wild-type mice survive for more than 30 days. In wild-type mice, expression of LRG-47 is induced on infection, in response to IFN-γ. The infected null mutants develop a profound anaemia by day 15, accompanied by alterations in splenic architecture, thrombocytopenia, lymphopenia and a dramatic depletion of bone marrow cellularity (Santiago *et al*. 2005). As well as these haematopoietic defects, macrophages derived from the null mutants also have a decreased ability to kill intracellular amastigotes (Santiago *et al*. 2005). The profound anaemia, and atrophy of spleen and bone marrow seen during infection of these null mice, suggest that LRG-47 plays a critical role in the control of haematopoiesis in infected animals and LRG-47 has recently been shown to be required for the correct response of haematopoietic stem cells to chemical or pathogenic insult (Feng *et al*. 2008). This suggests that the anaemia seen in some models of *T. cruzi* infection may be due to interference with the induction of LRG-47, leading to subsequent profound haematopietic defects.

The role of the macrophage iron-withholding response in either acute or chronic *T. cruzi* infection has not been studied. Given that *T. cruzi* replicates in the cytoplasm of macrophages it could be expected that such a response would benefit the parasite by allowing access to iron, unlike the situation with *T. brucei* and *Leishmania*.

IRON METABOLISM AS A DRUG TARGET

Iron is vital for all trypanosomatid parasites and plays a significant role in pathogenesis and immune control of these organisms. Depletion of this essential nutrient rapidly decreases the rate of DNA synthesis, increases oxidative stress levels via loss of SOD and APX activity, blocks J-base synthesis and stops electron transfer to the alternative oxidase, leading inexorably to death of the parasite. Iron chelation has been tested against all 3 groups of human pathogenic trypanosomatids. While iron chelation often has significant effects on parasites *in vitro*, during infection it is difficult to separate direct effects on the parasite from indirect effects mediated through the immune response. This may be particularly true for the intracellular trypanosomatids, *Leishmania* and *T. cruzi*.

In *T. brucei*, which one would expect to be especially sensitive as a bloodstream parasite, the iron chelator deferoxamine (DFO) has been shown, *in vitro*, to decrease growth rate, DNA synthesis and oxygen consumption, and has an IC_{50} of approximately 3·3 μM (Breidbach *et al*. 2002; Merschjohann and Steverding, 2006). These chelators act by iron sequestration, preventing the incorporation of iron into newly synthesized enzymes, rather than stripping iron from already active proteins. Decreases in growth rate and oxygen consumption are

almost entirely accounted for by inhibition of the alternative oxidase, rather than by a cumulative effect on all iron-dependent proteins. Fe-SOD activity is not affected by iron chelation, possibly due to the protein having a longer half-life. Surprisingly, there are no published studies of the effects of iron chelation on *T. brucei* infection in animal models.

DFO has been tested in animal models of *L. major* and *T. cruzi* infection. Using a cutaneous infection model (*L. major* in BALB/c mice), intraperitoneal injections of DFO have only a modest effect on lesion development. However, this may simply reflect the short half-life of DFO in plasma (5–10 min), with insufficient concentrations being achieved in the footpad. Surprisingly, when mice are given iron supplementation, in the form of intraperitoneal injections of iron-dextran, lesion development is significantly retarded. Mice injected with 8 mg iron dextran show no lesions up to 18 weeks after challenge (Bisti *et al.* 2000). The effect of iron supplementation appears to be due to its effect on the immune response of the host, rather than to any direct effect on the parasite. (Bisti *et al.* 2000; Bisti and Soteriadou, 2006). In this model, iron overloading leads to an increased and sustained infiltration of neutrophils and a strong oxidative burst during the initial phase of infection. In the later stages, there is activation of NF-κB and an increased number of IFN-γ positive $CD4^+$ T-cells are recruited to the draining lymph node. Thus, in this system iron probably mediates its effect via reactive oxygen species signalling through NF-κB, leading to a sustained TH1 response against the parasites (Bisti *et al.* 2006; Bisti and Soteriadou, 2006).

Experiments on iron depletion or overload in *T. cruzi*-infected mice have been carried out using DFO. In the case of the former the treated mice were also maintained on an iron-deficient diet to ensure their iron depleted status (Lalonde and Holbein, 1984). In these experiments, it was clear that *T. cruzi* infection is more severe and produces higher mortality in iron-overloaded mice than in iron-deficient mice. In a particularly susceptible mouse strain, C3H, lethality is reduced from 100% to 45% by DFO treatment. The time to death is also extended from 36·5 to 43·7 days for those that succumbed. A second study in which Swiss male mice were injected with DFO daily, also showed reduced parasite growth and mortality during the acute phase (Arantes *et al.* 2007). When the trypanocidal drug benznidazole is coupled with DFO, in the Swiss male mouse/*T. cruzi* Y strain model, the result is more effective than benznidazole alone, but only when the DFO treatment is started prior to infection. Since these animals are not maintained on an iron-deficient diet, pre-treatment was probably necessary to deplete iron levels sufficiently to make a difference to the parasite (Francisco *et al.* 2008). Nevertheless, as suggested by the interaction between SOD overexpression and benznidazole susceptibility (Temperton *et al.* 1998), modification of iron metabolism may potentiate the effects of other drugs against these parasites.

CONCLUDING REMARKS

Iron is essential for trypanosomatids, and the mechanism of acquisition and use are potential targets for new therapeutic approaches. These can be aimed at stimulating the host to kill the parasite or directly at the parasite itself. The last 30 years have seen a rapid increase in our knowledge of the biology of these parasites, but there are still many gaps in our understanding of the role of iron in the life cycle and pathogenesis of these organisms. This is especially the case with the American trypanosome *T. cruzi*, particularly the intracellular amastigote. We hope this review will go some way to stimulating further research into iron and trypanosomatids.

ACKNOWLEDGEMENTS

We thank Shane R. Wilkinson and Belinda S. Hall (Queen Mary, University of London) for critical reading of the manuscript, and Anne Koerber for help with the figures. We acknowledge the financial support of The Wellcome Trust (Grant No. 084175/Z/07/Z).

REFERENCES

Adak, S. and Datta, A. K. (2005). *Leishmania major* encodes an unusual peroxidase that is a close homologue of plant ascorbate peroxidase: a novel role of the transmembrane domain. *The Biochemical Journal* **390**, 465–474.

Ajayi, W. U., Chaudhuri, M. and Hill, G. C. (2002). Site-directed mutagenesis reveals the essentiality of the conserved residues in the putative diiron active site of the trypanosome alternative oxidase. *Journal of Biological Chemistry* **277**, 8187–8193.

Andersson, M. E. and Nordlund, P. (1999). A revised model of the active site of alternative oxidase. *FEBS Letters* **449**, 17–22.

Arantes, J. M., Pedrosa, M. L., Martins, H. R., Veloso, V. M., de Lana, M., Bahia, M. T., Tafuri, W. L. and Carneiro, C. M. (2007). *Trypanosoma cruzi*: treatment with the iron chelator desferrioxamine reduces parasitemia and mortality in experimentally infected mice. *Experimental Parasitology* **117**, 43–50.

Berthold, D. A. and Stenmark, P. (2003). Membrane-bound diiron carboxylate proteins. *Annu Rev Plant Biol* **54**, 497–517.

Bienen, E. J., Webster, P. and Fish, W. R. (1991). *Trypanosoma* (*Nannomonas*) *congolense*: changes in respiratory metabolism during the life cycle. *Experimental Parasitology* **73**, 403–412.

Biggs, T. E., Baker, S. T., Botham, M. S., Dhital, A., Barton, C. H. and Perry, V. H. (2001). Nramp1 modulates iron homoeostasis *in vivo* and *in vitro*: evidence for a role in cellular iron release involving

de-acidification of intracellular vesicles. *European Journal of Immunology* **31**, 2060–2070.

Bisti, S., Konidou, G., Boelaert, J., Lebastard, M. and Soteriadou, K. (2006). The prevention of the growth of *Leishmania major* progeny in BALB/c iron-loaded mice: a process coupled to increased oxidative burst, the amplitude and duration of which depend on initial parasite developmental stage and dose. *Microbes and Infection* **8**, 1464–1472.

Bisti, S., Konidou, G., Papageorgiou, F., Milon, G., Boelaert, J. R. and Soteriadou, K. (2000). The outcome of *Leishmania major* experimental infection in BALB/c mice can be modulated by exogenously delivered iron. *European Journal of Immunology* **30**, 3732–3740.

Bisti, S. and Soteriadou, K. (2006). Is the reactive oxygen species-dependent-NF-kappaB activation observed in iron-loaded BALB/c mice a key process preventing growth of *Leishmania major* progeny and tissue-damage? *Microbes and Infection* **8**, 1473–1482.

Bitter, W., Gerrits, H., Kieft, R. and Borst, P. (1998). The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. *Nature, London* **391**, 499–502.

Blackwell, J. M., Fakiola, M., Ibrahim, M. E., Jamieson, S. E., Jeronimo, S. B., Miller, E. N., Mishra, A., Mohamed, H. S., Peacock, C. S., Raju, M., Sundar, S. and Wilson, M. E. (2009). Genetics and visceral leishmaniasis: of mice and man. *Parasite Immunology* **31**, 254–266.

Borges, V. M., Vannier-Santos, M. A. and de Souza, W. (1998). Subverted transferrin trafficking in *Leishmania*-infected macrophages. *Parasitology Research* **84**, 811–822.

Breidbach, T., Scory, S., Krauth-Siegel, R. L. and Steverding, D. (2002). Growth inhibition of bloodstream forms of *Trypanosoma brucei* by the iron chelator deferoxamine. *International Journal for Parasitology* **32**, 473–479.

Bucheton, B., Abel, L., Kheir, M. M., Mirgani, A., El-Safi, S. H., Chevillard, C. and Dessein, A. (2003). Genetic control of visceral leishmaniasis in a Sudanese population: candidate gene testing indicates a linkage to the NRAMP1 region. *Genes Immun* **4**, 104–109.

Carvalho, S., Cruz, T., Santarem, N., Castro, H., Costa, V. and Tomas, A. M. (2009). Heme as a source of iron to *Leishmania infantum* amastigotes. *Acta Tropica* **109**, 131–135.

Chang, C. S. and Chang, K. P. (1985). Heme requirement and acquisition by extracellular and intracellular stages of *Leishmania mexicana* amazonensis. *Molecular and Biochemical Parasitology* **16**, 267–276.

Chaudhuri, M., Ott, R. D. and Hill, G. C. (2006). Trypanosome alternative oxidase: from molecule to function. *Trends in Parasitology* **22**, 484–491.

Chaudhuri, M., Sharan, R. and Hill, G. C. (2002). Trypanosome alternative oxidase is regulated post-transcriptionally at the level of RNA stability. *Journal of Eukaryotic Microbiology* **49**, 263–269.

Clarkson, A. B., Jr., Bienen, E. J., Pollakis, G. and Grady, R. W. (1989). Respiration of bloodstream forms of the parasite *Trypanosoma brucei brucei* is dependent on a plant-like alternative oxidase. *Journal of Biological Chemistry* **264**, 17770–17776.

Cliffe, L. J., Kieft, R., Southern, T., Birkeland, S. R., Marshall, M., Sweeney, K. and Sabatini, R. (2009). JBP1 and JBP2 are two distinct thymidine hydroxylases involved in J biosynthesis in genomic DNA of African trypanosomes. *Nucleic Acids Research* **37**, 1452–1462.

Condo, I., Ventura, N., Malisan, F., Rufini, A., Tomassini, B. and Testi, R. (2007). In vivo maturation of human frataxin. *Hum Mol Genet* **16**, 1534–1540.

Coustou, V., Besteiro, S., Biran, M., Diolez, P., Bouchaud, V., Voisin, P., Michels, P. A., Canioni, P., Baltz, T. and Bringaud, F. (2003). ATP generation in the *Trypanosoma brucei* procyclic form: cytosolic substrate level is essential, but not oxidative phosphorylation. *Journal of Biological Chemistry* **278**, 49625–49635.

d'Ieteren, G. D., Authie, E., Wissocq, N. and Murray, M. (1998). Trypanotolerance, an option for sustainable livestock production in areas at risk from trypanosomosis. *Rev Sci Tech* **17**, 154–175.

Das, N. K., Biswas, S., Solanki, S. and Mukhopadhyay, C. K. (2009). *Leishmania donovani* depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. *Cell Microbiol* **11**, 83–94.

Dolai, S., Yadav, R. K., Pal, S. and Adak, S. (2008). *Leishmania major* ascorbate peroxidase overexpression protects cells against reactive oxygen species-mediated cardiolipin oxidation. *Free Radic Biol Med* **45**, 1520–1529.

Dormeyer, M., Reckenfelderbaumer, N., Ludemann, H. and Krauth-Siegel, R. L. (2001). Trypanothione-dependent synthesis of deoxyribonucleotides by *Trypanosoma brucei* ribonucleotide reductase. *Journal of Biological Chemistry* **276**, 10602–10606.

Dormeyer, M., Schoneck, R., Dittmar, G. A. and Krauth-Siegel, R. L. (1997). Cloning, sequencing and expression of ribonucleotide reductase R2 from *Trypanosoma brucei*. *FEBS Letters* **414**, 449–453.

Dufernez, F., Yernaux, C., Gerbod, D., Noel, C., Chauvenet, M., Wintjens, R., Edgcomb, V. P., Capron, M., Opperdoes, F. R. and Viscogliosi, E. (2006). The presence of four iron-containing superoxide dismutase isozymes in trypanosomatidae: characterization, subcellular localization, and phylogenetic origin in *Trypanosoma brucei*. *Free Radic Biol Med* **40**, 210–225.

Dupuy, J., Volbeda, A., Carpentier, P., Darnault, C., Moulis, J. M. and Fontecilla-Camps, J. C. (2006). Crystal structure of human iron regulatory protein 1 as cytosolic aconitase. *Structure* **14**, 129–139.

Fairlamb, A. H., Henderson, G. B., Bacchi, C. J. and Cerami, A. (1987). In vivo effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **24**, 185–191.

Fast, B., Kremp, K., Boshart, M. and Steverding, D. (1999). Iron-dependent regulation of transferrin

receptor expression in *Trypanosoma brucei*. *The Biochemical Journal* **342**, 691–696.

Feng, C. G., Weksberg, D. C., Taylor, G. A., Sher, A. and Goodell, M. A. (2008). The p47 GTPase Lrg-47 (Irgm1) links host defense and hematopoietic stem cell proliferation. *Cell Stem Cell* **2**, 83–89.

Flohe, L., Hecht, H. J. and Steinert, P. (1999). Glutathione and trypanothione in parasitic hydroperoxide metabolism. *Free Radic Biol Med* **27**, 966–984.

Francisco, A. F., de Abreu Vieira, P. M., Arantes, J. M., Pedrosa, M. L., Martins, H. R., Silva, M., Veloso, V. M., de Lana, M., Bahia, M. T., Tafuri, W. L. and Carneiro, C. M. (2008). *Trypanosoma cruzi*: effect of benznidazole therapy combined with the iron chelator desferrioxamine in infected mice. *Experimental Parasitology* **120** 314–319.

Genest, P. A., ter Riet, B., Dumas, C., Papadopoulou, B., van Luenen, H. G. and Borst, P. (2005). Formation of linear inverted repeat amplicons following targeting of an essential gene in *Leishmania*. *Nucleic Acids Research* **33**, 1699–1709.

Govoni, G., Vidal, S., Gauthier, S., Skamene, E., Malo, D. and Gros, P. (1996). The Bcg/Ity/Lsh locus: genetic transfer of resistance to infections in C57BL/6J mice transgenic for the Nramp1 Gly169 allele. *Infection and Immunity* **64**, 2923–2929.

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I. and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **119**, 753–766.

Hall, B. S., Gabernet-Castello, C., Voak, A., Goulding, D., Natesan, S. K. and Field, M. C. (2006). TbVps34, the trypanosome orthologue of Vps34, is required for Golgi complex segregation. *Journal of Biological Chemistry* **281**, 27600–27612.

Hall, B. S., Smith, E., Langer, W., Jacobs, L. A., Goulding, D. and Field, M. C. (2005). Developmental variation in Rab11-dependent trafficking in *Trypanosoma brucei*. *Eukaryotic Cell* **4**, 971–980.

Halliwell, B. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*, 4th Edn. Oxford University Press, Oxford, UK.

Helfert, S., Estevez, A. M., Bakker, B., Michels, P. and Clayton, C. (2001). Roles of triosephosphate isomerase and aerobic metabolism in *Trypanosoma brucei*. *The Biochemical Journal* **357**, 117–125.

Hiner, A. N., Martinez, J. I., Arnao, M. B., Acosta, M., Turner, D. D., Lloyd Raven, E. and Rodriguez-Lopez, J. N. (2001). Detection of a tryptophan radical in the reaction of ascorbate peroxidase with hydrogen peroxide. *European Journal of Biochemistry* **268**, 3091–3098.

Hofer, A., Schmidt, P. P., Graslund, A. and Thelander, L. (1997). Cloning and characterization of the R1 and R2 subunits of ribonucleotide reductase from *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences, USA* **94**, 6959–6964.

Horvath, A., Horakova, E., Dunajcikova, P., Verner, Z., Pravdova, E., Slapetova, I., Cuninkova, L. and Lukes, J. (2005). Downregulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic *Trypanosoma brucei*. *Molecular Microbiology* **58**, 116–130.

Huynh, C., Sacks, D. L. and Andrews, N. W. (2006). A *Leishmania amazonensis* ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. *Journal of Experimental Medicine* **203**, 2363–2375.

Irigoin, F., Cibils, L., Comini, M. A., Wilkinson, S. R., Flohe, L. and Radi, R. (2008). Insights into the redox biology of *Trypanosoma cruzi*: Trypanothione metabolism and oxidant detoxification. *Free Radic Biol Med* **45**, 733–742.

Isobe, T., Holmes, E. C. and Rudenko, G. (2003). The transferrin receptor genes of *Trypanosoma equiperdum* are less diverse in their transferrin binding site than those of the broad-host range *Trypanosoma brucei*. *Journal of Molecular Evolution* **56**, 377–386.

Jabado, N., Cuellar-Mata, P., Grinstein, S. and Gros, P. (2003). Iron chelators modulate the fusogenic properties of Salmonella-containing phagosomes. *Proceedings of the National Academy of Sciences, USA* **100**, 6127–6132.

Jeffries, T. R., Morgan, G. W. and Field, M. C. (2001). A developmentally regulated rab11 homologue in *Trypanosoma brucei* is involved in recycling processes. *Journal of Cell Science* **114**, 2617–2626.

Kitajima, S., Kurioka, M., Yoshimoto, T., Shindo, M., Kanaori, K., Tajima, K. and Oda, K. (2008). A cysteine residue near the propionate side chain of heme is the radical site in ascorbate peroxidase. *FEBS J* **275**, 470–480.

Krauth-Siegel, R. L. and Ludemann, H. (1996). Reduction of dehydroascorbate by trypanothione. *Mololecular and Biochemical Parasitology* **80**, 203–208.

Krishnamurthy, G., Vikram, R., Singh, S. B., Patel, N., Agarwal, S., Mukhopadhyay, G., Basu, S. K. and Mukhopadhyay, A. (2005). Hemoglobin receptor in *Leishmania* is a hexokinase located in the flagellar pocket. *Journal of Biological Chemistry* **280**, 5884–5891.

Lalonde, R. G. and Holbein, B. E. (1984). Role of iron in *Trypanosoma cruzi* infection of mice. *Journal of Clinical Investigation* **73**, 470–476.

Lara, F. A., Sant'anna, C., Lemos, D., Laranja, G. A., Coelho, M. G., Reis Salles, I., Michel, A., Oliveira, P. L., Cunha, E. S. N., Salmon, D. and Paes, M. C. (2007). Heme requirement and intracellular trafficking in *Trypanosoma cruzi* epimastigotes. *Biochemical and Biophysical Research Communications* **355**, 16–22.

Ligtenberg, M. J., Bitter, W., Kieft, R., Steverding, D., Janssen, H., Calafat, J. and Borst, P. (1994). Reconstitution of a surface transferrin binding complex in insect form *Trypanosoma brucei*. *EMBO Journal* **13**, 2565–2573.

Lima, M. F. and Villalta, F. (1990). *Trypanosoma cruzi* receptors for human transferrin and their role. *Molecular and Biochemical Parasitology* **38**, 245–252.

Long, S., Jirku, M., Ayala, F. J. and Lukes, J. (2008*a*). Mitochondrial localization of human frataxin is necessary but processing is not for rescuing frataxin deficiency in *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences, USA* **105**, 13468–13473.

Long, S., Jirku, M., Mach, J., Ginger, M. L., Sutak, R., Richardson, D., Tachezy, J. and Lukes, J. (2008*b*).

Ancestral roles of eukaryotic frataxin: mitochondrial frataxin function and heterologous expression of hydrogenosomal *Trichomonas* homologues in trypanosomes. *Molecular Microbiology* **69**, 94–109.

MacMicking, J. D. (2005). Immune control of phagosomal bacteria by p47 GTPases. *Current Opinion in Microbiology* **8**, 74–82.

Maier, A. and Steverding, D. (1996). Low affinity of *Trypanosoma brucei* transferrin receptor to apotransferrin at pH 5 explains the fate of the ligand during endocytosis. *FEBS Letters* **396**, 87–89.

Marcondes, M. C., Borelli, P., Yoshida, N. and Russo, M. (2000). Acute *Trypanosoma cruzi* infection is associated with anemia, thrombocytopenia, leukopenia, and bone marrow hypoplasia: reversal by nifurtimox treatment. *Microbes and Infection* **2**, 347–352.

Mauricio, I. L., Stothard, J. R. and Miles, M. A. (2000). The strange case of *Leishmania chagasi*. *Parasitology Today* **16**, 188–189.

Mehta, A. and Shaha, C. (2006). Mechanism of metalloid-induced death in *Leishmania* spp.: role of iron, reactive oxygen species, Ca2+, and glutathione. *Free Radic Biol Med* **40**, 1857–1868.

Merschjohann, K. and Steverding, D. (2006). In vitro growth inhibition of bloodstream forms of *Trypanosoma brucei* and *Trypanosoma congolense* by iron chelators. *Kinetoplastid Biol Dis* **5**, 3.

Mohamed, H. S., Ibrahim, M. E., Miller, E. N., White, J. K., Cordell, H. J., Howson, J. M., Peacock, C. S., Khalil, E. A., El Hassan, A. M. and Blackwell, J. M. (2004). SLC11A1 (formerly NRAMP1) and susceptibility to visceral leishmaniasis in The Sudan. *European Journal of Human Genetics* **12**, 66–74.

Morgan, G. W., Allen, C. L., Jeffries, T. R., Hollinshead, M. and Field, M. C. (2001). Developmental and morphological regulation of clathrin-mediated endocytosis in *Trypanosoma brucei*. *Journal of Cell Science* **114**, 2605–2615.

Mussmann, R., Engstler, M., Gerrits, H., Kieft, R., Toaldo, C. B., Onderwater, J., Koerten, H., van Luenen, H. G. and Borst, P. (2004). Factors affecting the level and localization of the transferrin receptor in *Trypanosoma brucei*. *Journal of Biological Chemistry* **279**, 40690–40698.

Mussmann, R., Janssen, H., Calafat, J., Engstler, M., Ansorge, I., Clayton, C. and Borst, P. (2003). The expression level determines the surface distribution of the transferrin receptor in *Trypanosoma brucei*. *Molecular Microbiology* **47**, 23–35.

Naessens, J. (2006). Bovine trypanotolerance: A natural ability to prevent severe anaemia and haemophagocytic syndrome? *International Journal for Parasitology* **36**, 521–528.

Nemeth, E., Preza, G. C., Jung, C. L., Kaplan, J., Waring, A. J. and Ganz, T. (2006). The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. *Blood* **107**, 328–333.

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T. and Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306**, 2090–2093.

O'Brien, T. C., Mackey, Z. B., Fetter, R. D., Choe, Y., O'Donoghue, A. J., Zhou, M., Craik, C. S., Caffrey, C. R. and McKerrow, J. H. (2008). A parasite cysteine protease is key to host protein degradation and iron acquisition. *Journal of Biological Chemistry* **283**, 28934–28943.

Pal, A., Hall, B. S., Jeffries, T. R. and Field, M. C. (2003). Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in *Trypanosoma brucei*. *The Biochemical Journal* **374**, 443–451.

Peyssonnaux, C., Zinkernagel, A. S., Datta, V., Lauth, X., Johnson, R. S. and Nizet, V. (2006). TLR4-dependent hepcidin expression by myeloid cells in response to bacterial pathogens. *Blood* **107**, 3727–3732.

Piacenza, L., Irigoin, F., Alvarez, M. N., Peluffo, G., Taylor, M. C., Kelly, J. M., Wilkinson, S. R. and Radi, R. (2007). Mitochondrial superoxide radicals mediate programmed cell death in *Trypanosoma cruzi*: cytoprotective action of mitochondrial iron superoxide dismutase overexpression. *The Biochemical Journal* **403**, 323–334.

Plewes, K. A., Barr, S. D. and Gedamu, L. (2003). Iron superoxide dismutases targeted to the glycosomes of *Leishmania chagasi* are important for survival. *Infection and Immunity* **71**, 5910–5920.

Prathalingham, S. R., Wilkinson, S. R., Horn, D. and Kelly, J. M. (2007). Deletion of the *Trypanosoma brucei* superoxide dismutase gene sodb1 increases sensitivity to nifurtimox and benznidazole. *Antimicrobial Agents and Chemotherapy* **51**, 755–758.

Radtke, A. L. and O'Riordan, M. X. (2006). Intracellular innate resistance to bacterial pathogens. *Cell Microbiol* **8**, 1720–1729.

Salahudeen, A. A., Thompson, J. W., Ruiz, J. C., Ma, H. W., Kinch, L. N., Li, Q., Grishin, N. V. and Bruick, R. K. (2009). An E3 ligase possessing an iron responsive hemerythrin domain is a regulator of iron homeostasis. *Science* **326**, 722–726.

Salmon, D., Geuskens, M., Hanocq, F., Hanocq-Quertier, J., Nolan, D., Ruben, L. and Pays, E. (1994). A novel heterodimeric transferrin receptor encoded by a pair of VSG expression site-associated genes in *T. brucei*. *Cell* **78**, 75–86.

Salmon, D., Hanocq-Quertier, J., Paturiaux-Hanocq, F., Pays, A., Tebabi, P., Nolan, D. P., Michel, A. and Pays, E. (1997). Characterization of the ligand-binding site of the transferrin receptor in *Trypanosoma brucei* demonstrates a structural relationship with the N-terminal domain of the variant surface glycoprotein. *EMBO Journal* **16**, 7272–7278.

Salmon, D., Paturiaux-Hanocq, F., Poelvoorde, P., Vanhamme, L. and Pays, E. (2005). *Trypanosoma brucei*: growth differences in different mammalian sera are not due to the species-specificity of transferrin. *Experimental Parasitology* **109**, 188–194.

Santiago, H. C., Feng, C. G., Bafica, A., Roffe, E., Arantes, R. M., Cheever, A., Taylor, G., Vieira, L. Q., Aliberti, J., Gazzinelli, R. T. and Sher, A. (2005). Mice deficient in LRG-47 display enhanced susceptibility to *Trypanosoma cruzi* infection associated with defective hemopoiesis and intracellular

control of parasite growth. *Journal of Immunology* **175**, 8165–8172.

Schaible, U. E. and Kaufmann, S. H. (2004). Iron and microbial infection. *Nat Rev Microbiol* **2**, 946–953.

Schell, D., Borowy, N. K. and Overath, P. (1991*a*). Transferrin is a growth factor for the bloodstream form of *Trypanosoma brucei*. *Parasitology Research* **77**, 558–560.

Schell, D., Evers, R., Preis, D., Ziegelbauer, K., Kiefer, H., Lottspeich, F., Cornelissen, A. W. and Overath, P. (1991*b*). A transferrin-binding protein of *Trypanosoma brucei* is encoded by one of the genes in the variant surface glycoprotein gene expression site. *EMBO Journal* **10**, 1061–1066.

Schofield, C. J. and Zhang, Z. (1999). Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes. *Curr Opin Struct Biol* **9**, 722–731.

Schwartz, K. J. and Bangs, J. D. (2007). Regulation of protein trafficking by glycosylphosphatidylinositol valence in African trypanosomes. *Journal of Eukaryotic Microbiology* **54**, 22–24.

Schwartz, K. J., Peck, R. F., Tazeh, N. N. and Bangs, J. D. (2005). GPI valence and the fate of secretory membrane proteins in African trypanosomes. *Journal of Cell Science* **118**, 5499–5511.

Smid, O., Horakova, E., Vilimova, V., Hrdy, I., Cammack, R., Horvath, A., Lukes, J. and Tachezy, J. (2006). Knock-downs of iron-sulfur cluster assembly proteins IscS and IscU down-regulate the active mitochondrion of procyclic *Trypanosoma brucei*. *Journal of Biological Chemistry* **281**, 28679–28686.

Soares, M. J. and de Souza, W. (1991). Endocytosis of gold-labeled proteins and LDL by *Trypanosoma cruzi*. *Parasitology Research* **77**, 461–468.

Soares, M. J., Souto-Padron, T. and De Souza, W. (1992). Identification of a large pre-lysosomal compartment in the pathogenic protozoon *Trypanosoma cruzi*. *Journal of Cell Science* **102**, 157–167.

Srivastava, P., Sharma, G. D., Kamboj, K. K., Rastogi, A. K. and Pandey, V. C. (1997). Heme metabolism in promastigotes of *Leishmania donovani*. *Molecular and Cellular Biochemistry* **171**, 65–68.

Steverding, D. (1998). Bloodstream forms of *Trypanosoma brucei* require only small amounts of iron for growth. *Parasitol Research* **84**, 59–62.

Steverding, D. (2003). The significance of transferrin receptor variation in *Trypanosoma brucei*. *Trends in Parasitology* **19**, 125–127.

Steverding, D. (2006). On the significance of host antibody response to the *Trypanosoma brucei* transferrin receptor during chronic infection. *Microbes and Infection* **8**, 2777–2782.

Steverding, D., Stierhof, Y. D., Chaudhri, M., Ligtenberg, M., Schell, D., Beck-Sickinger, A. G. and Overath, P. (1994). ESAG 6 and 7 products of *Trypanosoma brucei* form a transferrin binding protein complex. *European Journal of Cell Biology* **64**, 78–87.

Steverding, D., Stierhof, Y. D., Fuchs, H., Tauber, R. and Overath, P. (1995). Transferrin-binding protein complex is the receptor for transferrin uptake in *Trypanosoma brucei*. *Journal of Cell Biology* **131**, 1173–1182.

Stijlemans, B., Vankrunkelsven, A., Brys, L., Magez, S. and De Baetselier, P. (2008). Role of iron homeostasis in trypanosomiasis-associated anemia. *Immunobiology* **213**, 823–835.

Taylor, M. C. and Kelly, J. M. (2006). pTcINDEX: a stable tetracycline-regulated expression vector for *Trypanosoma cruzi*. *BMC Biotechnol* **6**, 32.

Temperton, N. J., Wilkinson, S. R., Meyer, D. J. and Kelly, J. M. (1998). Overexpression of superoxide dismutase in *Trypanosoma cruzi* results in increased sensitivity to the trypanocidal agents gentian violet and benznidazole. *Molecular and Biochemical Parasitology* **96**, 167–176.

Theodoropoulos, T. A., Silva, A. G. and Bestetti, R. B. (2009). Eosinophil blood count and anemia are associated with *Trypanosoma cruzi* infection reactivation in Chagas' heart transplant recipients. *Int J Cardiol* (in the Press).

Vainio, S., Genest, P. A., ter Riet, B., van Luenen, H. and Borst, P. (2009). Evidence that J-binding protein 2 is a thymidine hydroxylase catalyzing the first step in the biosynthesis of DNA base J. *Molecular and Biochemical Parasitology* **164**, 157–161.

van Luenen, H. G., Kieft, R., Mussmann, R., Engstler, M., ter Riet, B. and Borst, P. (2005). Trypanosomes change their transferrin receptor expression to allow effective uptake of host transferrin. *Molecular Microbiology* **58**, 151–165.

Vashisht, A. A., Zumbrennen, K. B., Huang, X., Powers, D. N., Durazo, A., Sun, D., Bhaskaran, N., Persson, A., Uhlen, M., Sangfelt, O., Spruck, C., Leibold, E. A. and Wohlschlegel, J. A. (2009). Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science* **326**, 718–721.

Vidal, S., Tremblay, M. L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., Skamene, E., Olivier, M., Jothy, S. and Gros, P. (1995). The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *Journal of Experimental Medicine* **182**, 655–666.

Voyiatzaki, C. S. and Soteriadou, K. P. (1992). Identification and isolation of the *Leishmania* transferrin receptor. *Journal of Biological Chemistry* **267**, 9112–9117.

Walden, W. E., Selezneva, A. I., Dupuy, J., Volbeda, A., Fontecilla-Camps, J. C., Theil, E. C. and Volz, K. (2006). Structure of dual function iron regulatory protein 1 complexed with ferritin IRE-RNA. *Science* **314**, 1903–1908.

Wallander, M. L., Leibold, E. A. and Eisenstein, R. S. (2006). Molecular control of vertebrate iron homeostasis by iron regulatory proteins. *Biochimica Biophysica Acta* **1763**, 668–689.

Wilkinson, S. R. and Kelly, J. M. (2003). The role of glutathione peroxidases in trypanosomatids. *Biological Chemistry* **384**, 517–525.

Wilkinson, S. R., Obado, S. O., Mauricio, I. L. and Kelly, J. M. (2002*a*). *Trypanosoma cruzi* expresses a plant-like ascorbate-dependent hemoperoxidase localized to the endoplasmic reticulum. *Proceedings of the National Academy of Sciences, USA* **99**, 13453–13458.

Wilkinson, S. R., Prathalingam, S. R., Taylor, M. C., Ahmed, A., Horn, D. and Kelly, J. M. (2006).

Functional characterisation of the iron superoxide dismutase gene repertoire in *Trypanosoma brucei*. *Free Radic Biol Med* **40**, 198–209.

Wilkinson, S. R., Taylor, M. C., Touitha, S., Mauricio, I. L., Meyer, D. J. and Kelly, J. M. (2002*b*). TcGPXII, a glutathione-dependent *Trypanosoma cruzi* peroxidase with substrate specificity restricted to fatty acid and phospholipid hydroperoxides, is localized to the endoplasmic reticulum. *The Biochemical Journal* **364**, 787–794.

Wilkinson, S. R., Temperton, N. J., Mondragon, A. and Kelly, J. M. (2000). Distinct mitochondrial and cytosolic enzymes mediate trypanothione-dependent peroxide metabolism in *Trypanosoma cruzi*. *Journal of Biological Chemistry* **275**, 8220–8225.

Wilson, M. E., Lewis, T. S., Miller, M. A., McCormick, M. L. and Britigan, B. E. (2002). *Leishmania chagasi*: uptake of iron bound to lactoferrin or transferrin requires an iron reductase. *Experimental Parasitology* **100**, 196–207.

Wilson, M. E., Vorhies, R. W., Andersen, K. A. and Britigan, B. E. (1994). Acquisition of iron from transferrin and lactoferrin by the protozoan *Leishmania chagasi*. *Infection and Immunity* **62**, 3262–3269.

Witola, W. H., Sarataphan, N., Inoue, N., Ohashi, K. and Onuma, M. (2005). Genetic variability in ESAG6 genes among *Trypanosoma evansi* isolates and in comparison to other Trypanozoon members. *Acta Tropica* **93**, 63–73.

Yabu, Y., Minagawa, N., Kita, K., Nagai, K., Honma, M., Sakajo, S., Koide, T., Ohta, N. and Yoshimoto, A. (1998). Oral and intraperitoneal treatment of *Trypanosoma brucei brucei* with a combination of ascofuranone and glycerol in mice. *Parasitology International* **47**, 131–137.

Yabu, Y., Suzuki, T., Nihei, C., Minagawa, N., Hosokawa, T., Nagai, K., Kita, K. and Ohta, N. (2006). Chemotherapeutic efficacy of ascofuranone in *Trypanosoma vivax*-infected mice without glycerol. *Parasitology International* **55**, 39–43.

Yabu, Y., Yoshida, A., Suzuki, T., Nihei, C., Kawai, K., Minagawa, N., Hosokawa, T., Nagai, K., Kita, K. and Ohta, N. (2003). The efficacy of ascofuranone in a consecutive treatment on *Trypanosoma brucei brucei* in mice. *Parasitology International* **52**, 155–164.

Yu, Z., Genest, P. A., ter Riet, B., Sweeney, K., DiPaolo, C., Kieft, R., Christodoulou, E., Perrakis, A., Simmons, J. M., Hausinger, R. P., van Luenen, H. G., Rigden, D. J., Sabatini, R. and Borst, P. (2007). The protein that binds to DNA base J in trypanosomatids has features of a thymidine hydroxylase. *Nucleic Acids Research* **35**, 2107–2115.