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Withstanding the challenges of host immunity: antigenic variation and the trypanosome surface coat

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Abstract

Prolonged survival in the face of host immunity has been a major force shaping the biology and evolution of the African trypanosomes, and nowhere are the effects of this force more apparent than in the antigenic variation of the trypanosome variant surface glycoprotein (VSG) coat. The coat protects the trypanosome within it from immune effectors, and spontaneous and stochastic events occurring at the molecular level cause individual trypanosomes to change the VSG variant they are expressing. The consequence of this switching at the population level is a diverse population that can pre-empt the specific immune responses that arise against VSG. The template for changes to VSG is an extensive archive of silent *VSG* genes and pseudogenes. *VSG* from the archive are activated not only as full-length genes but also through the combination of segments to form mosaic *VSG* genes, a process that augments the potential for antigenic variation by introducing combinatorial variation and allowing *VSG* pseudogenes to be used. The main part of the archive occupies subtelomeres and so is itself prone to mutation and rapid evolution, which are important features when superinfection or reinfection of partially-immune hosts is necessary. The antigenic variation 'diversity phenotype' is thus a multifaceted one, enlisting and coordinating fundamental mechanisms of cell biology to bring about a process that unfolds across populations, thereby facilitating the success of the African trypanosomes.

Introduction: Antigenic variation is a diversity phenotype key to the African trypanosomes' success

The success of African salivarian trypanosomes depends on their survival in mammalian blood. It is only blood-borne trypanosomes that can be transmitted by biting insects, and so traits prolonging persistence in this habitat are likely to be

beneficial, because they increase opportunities for transmission. Blood is chemically stable and rich in nutrients but is intensely hostile to foreign bodies, thanks to a sophisticated immune system that deploys both immediate, non-specific 'innate' responses, and specific 'adaptive' responses to efficiently eradicate pathogens. Unusually amongst chronic pathogens, African trypanosomes do not invade host cells to escape from immunity. Instead, they are extracellular during the mammalian phase of their lifecycle. Protecting each parasite from host immunity is a dense, uniform, glycoprotein 'coat' that covers its entire surface, made from approximately six million dimers (Jackson et al. 1985) of the major African trypanosome variable antigen: variant surface glycoprotein (VSG).

The VSG surface coat is critical for a trypanosome to survive in the blood—in its absence or dysfunction, parasites are killed by factors present in naive serum (Ferrante and Allison 1983; Mosser and Roberts 1982). An intact VSG coat obstructs immune effectors, preventing them from accessing the cell membrane or invariant surface antigens (Overath et al. 1994), and in doing so it forms the most immediate interface between parasite and host during infection. Although the coat shields the parasite surface, VSG itself is highly immunogenic, and adaptive responses against the antigen are rapidly mounted by the host (Black et al. 2010). A mechanism to withstand the challenges of adaptive immunity operates at the population level: a single host can maintain a population of millions, if not billions, of trypanosomes (Barry 1986), and as an individual trypanosome multiplies, it can switch to express a different VSG. The diversity that unfolds across the burgeoning population pre-empts specific immune responses, making the infection resilient to eradication. As different VSG variants are targeted and neutralised by immunity, the population is replenished by the proliferation of the survivors, which undergo further VSG switches and are further shaped and selected by immunity to yield the patterns of antigenic variation of which the African trypanosomes are a paradigm. The VSG coat—and its variation—represents a remarkable example of biological diversity that harnesses genome biology, cellular processes and population dynamics to enable African trypanosomes to survive despite a precarious lifecycle that is balanced between a broad range of host mammals and the insects that feed on them.

Compared with general mutation of antigens caused by background cellular processes, true antigenic variation is a system that has evolved under pressure from adaptive immunity to present an ever-changing 'diversity phenotype'. With the African trypanosomes, we can clearly infer that such selection has occurred by identifying distinct processes and resources specifically associated with introducing dramatic change in the expressed antigen: (i) exclusive, tightly-regulated expression of VSG from a dedicated locus; (ii) mechanisms to vary the expressed *VSG* gene; (iii) an archive of silent *VSG* genes that can be utilised as source material for this variation. Importantly, the antigenic variation diversity phenotype is a program emerging from a trypanosome *genome*, one that becomes manifest in the behaviour of a population of clonally related parasites. Selection favours those genomes that maximise their overall transmission and propagation, and strategies that might appear counterintuitive or hugely wasteful in terms of the death of individual parasites can be highly effective at maximising the transmission, and hence success, of the genomes that give rise to those strategies (Reece et al. 2011). Similar examples abound across biology (West et al. 2006), one being the transmission of cellular slime moulds such as *Dictyostelium discoidium*. Upon starvation, unicellular *D. discoidium* amoebae unite to form a fruiting body, a process that is necessary for the effective dispersal of spores. However, only some cells are transmitted, the others perish in the construction of the fruiting body, losses which are accommodated—indeed, selected for—at the population level (Hudson et al. 2002). An understanding of the multiple levels at which selection acts means we must consider not only the specific molecular events that occur within individual trypanosomes during infection, but also the behaviours of the trypanosome population that emerge from a genome's program (Marcello and Barry 2007b), and how the patterns of diversity that appear interact with other key pressures on trypanosome biology and natural history.

In this chapter we will discuss the antigenic variation phenotype: the molecular processes that underpin it, and the highly successful survival strategy that emerges. First, the processes occurring in individual trypanosomes will be covered. Then we will discuss how these features play out over the course of infection. The broader evolutionary trajectories of the genes involved will then be discussed, followed by an overview of how this phenotype might interact with other key features of the trypanosomes' natural histories. Finally, we will consider the outstanding questions

posed by antigenic variation, and what approaches might be best suited to addressing them.

Although all species of African salivarian trypanosome possess VSG, undergo antigenic variation and are subject to the pressures imposed by mammalian adaptive immunity, it is likely that differences exist in the patterns and structures involved. This chapter will focus on the model organism *Trypanosoma brucei*, which is by far the best characterised. Features of the *T. brucei* antigenic variation system are likely to apply also in the T. brucei derivatives *T. evansi* and *T. equiperdum*. Where data are sufficient, related species such as *T. congolense* and *T. vivax* will be covered, but the details of antigenic variation in these parasites are less clear.

How does the structure of the VSG coat relate to its function?

At the level of the individual, a key feature of VSG is its ability to form an effective barrier. VSG must therefore possess a particular structure, one that confers depth and density on an intact coat. Yet at the level of the population, different VSG coats must vary sufficiently from one another to enable antigenic variation. These demands have been met by a conserved tertiary fold that can be formed by widely divergent VSG amino acid sequences (Blum et al. 1993; Carrington et al. 1991). In this way, functional barrier-forming variants can be completely antigenically distinct from one another, allowing evasion of both non-specific innate immunity and specific adaptive immunity.

The VSG dimer forms an elongated structure that stands perpendicular to the membrane, the overall effect being a deep coat within which invariant surface molecules are buried (Vickerman 1969), as shown in Figure 1. The VSG N-terminal domain (NTD) forms the exposed, membrane-distal part of the antigen (Schwede et al. 2011). This domain is essentially a pair of long helical elements that mediate dimerisation (Cohen et al. 1984) , from which hang numerous loops and smaller units of secondary structure. In the case of the *brucei* clade, VSG also possesses a C-terminal domain (CTD) comprising one or two small subdomains, which may act to extend the molecule further and increase packing density of the coat (Chattopadhyay et al. 2005; Jones et al. 2008), but are apparently absent from *T. congolense* and *T. vivax* (Jackson et al. 2012). Both the NTD and the CTD are reinforced by a number of disulphide bridges between highly conserved cysteine residues (Allen and Gurnett

1983; Bussler et al. 1998); these probably strengthen the molecule against the vigorous physical forces it is likely to encounter (O'Beirne et al. 1998). VSG are anchored in the membrane by means of a glycosylphosphatidylinositol (GPI) group, which can be distinguished from the mammalian host GPI anchors by its shorter dimyristoylglycerol lipid moiety, and (for most VSG) its possession of galactose residues (Ferguson 1991); and VSG are often also N-glycosylated elsewhere (Mehlert et al. 2002). Glycosylation may enhance the barrier function of an intact surface coat, and contribute to the structure and accessibility of epitopes, as it does for many other variable antigens such as influenza haemagglutinin (Caton et al. 1982). The lack of a transmembrane domain results in a coat that is exceptionally fluid: VSG dimers can flex and spin in place, move freely across the surface of the parasite (Engstler et al. 2007), and constitutively recycle through the endosomal system (Pal et al. 2003; Seyfang et al. 1990), creating a bustling, dynamic structure. This fluidity may facilitate efficient function of other surface molecules such as transferrin (Pal et al. 2003), and may assist in the rapid turnover of the coat that occurs during switching. There is also evidence that coat fluidity can assist the clearance of surface bound antibodies when these are present at low concentration, providing a further, complementary means of immune escape (Engstler et al. 2007).

VSG production is a tightly regulated process

It is presumably important that parasites maintain coat density whilst undergoing growth and replication, and VSG production is a principal aspect of the biology of the proliferating bloodstream form trypanosome (Smith et al. 2009). The large surface area covered by an intact coat requires the production of huge quantities of the coat protein—in fact, approximately 10% of *T. brucei* total soluble protein is VSG (Cross 1990). VSG is transcribed from a specialised locus, the active expression site (ES), one reason for which may be the requirement for a large volume of VSG mRNA required by such high expression. There are two types of expression site, metacyclic (MES), of which there are approximately 20 in a *T. brucei* genome (Horn and Barry 2005), and bloodstream (BES), which number up to 23 (Young et al. 2008). Each ES contains a single intact *VSG* gene and a number of expression site associated genes (ESAGs) (McCulloch and Horn 2009); several ESAGs have functions that have been experimentally associated with survival in the mammalian blood, such as iron acquisition (Bitter et al. 1998) and immune system modulation (Salmon et al. 2012),

and ESAG content varies between expression sites (Hertz-Fowler et al. 2008). In fact MES possess no ESAGs at all, and thus metacyclic *VSG* are unique in being the only trypanosome genes transcribed from their own promoter (Graham and Barry 1995). Critically, the expression sites are under strict control: only one is active at any time. The actively transcribed VSG itself is at the promoter-distal end of the expression site, proximal to the telomere repeats (Aline and Stuart 1989). Upstream of the *VSG*, and separating it from the rest of the expression site, is a set of 70-bp AT-rich imperfect repeats (Campbell et al. 1984). Transcription of the active expression site is initiated at a single promoter, and separate mRNA molecules for each of the genes in the ES are produced by trans-splicing of this polycistronic transcript with a 5' 'spliced leader' sequence that is common to all trypanosome mRNAs (Cully et al. 1985; Parsons et al. 1984). Newly translated VSG is folded in the endoplasmic reticulum with the aid of numerous chaperones (Field et al. 2010) and undergoes post-translational modification such as signal peptide cleavage (McConnell et al. 1981) and glycosylation (Ferguson et al. 1986; Mehlert et al. 2002). Mature VSG reaches the surface at the 'flagellar pocket', an invagination of the plasma membrane at the base of the flagellum where all endocytosis and exocytosis takes place (Overath et al. 1997), from where it diffuses to spread across the entire parasite surface.

Only one VSG is transcribed at a time

Because each ES contains just one intact *VSG*, and because just one ES is active at any time, each individual trypanosome transcribes only one *VSG*. Exclusive expression is thought to be a strongly selected trait: simultaneous expression of multiple *VSG* would result in a heterogeneous coat, exposing all of the expressed VSG to the immune system, and resulting in a shorter infection than could have been achieved, had the different variants been expressed exclusively and consecutively (Morrison et al. 2005). An example of the importance of exclusive expression in prolonging infection can be found in another antigenically variant pathogen, *Giardia lamblia*, where disruption of monoallelic expression resulted in immune responses developing against all exposed antigens (Rivero et al. 2010). It has proved possible to generate parasites that simultaneously express two different VSG from one expression site (Muñoz-Jordán et al. 1996), a result indicating that the presence of just one *VSG* in an expression site is most likely a consequence of the switching mechanism rather than a demand enforced by it. On the other hand, the exclusive activation of a single

ES—monoallelic expression—is much more strictly maintained, and attempts to generate lines expressing multiple ES result only in unstable clones switching rapidly between ES (Ulbert et al. 2002). How is it that one, and only one, ES is active at a time? One hypothesis has been built on the identification of a single multi-component complex that drives transcriptional elongation from the active ES, termed the expression site body (ESB) (Navarro and Gull 2001). The ESB associates closely with the mitotic machinery, which could explain the heritability of the exclusive ESB-ES association (Landeira et al. 2009). However, the presence of a single ESB could be taken as a consequence of monoallelic expression as much as its cause and there are many questions about the mechanism that remain unclear, not least how a single ESB is maintained (Horn and McCulloch 2010). At the same time, complementary processes silence the inactive ESs, and a number of epigenetic control pathways have been implicated (Alsford et al. 2012). There are numerous factors for which depletion has been shown to allow some transcription from inactive ES, including chromatin remodelling factors (Hughes et al. 2007; Wang et al. 2010), histones (Povelones et al. 2012) and histone chaperones (Alsford et al. 2012), and DNA replication factors (Benmerzouga et al. 2013; Tiengwe et al. 2012), to list only a few. However, in no case has derepression led to a level of transcription comparable to that from the active ES. The physical location of inactive ES at the nuclear periphery has also been proposed to contribute to their repression (DuBois et al. 2012; Navarro et al. 2007). The number of candidate factors associated with monoallelic expression, alongside the close physical proximity of these systems in a living cell, raises the possibility that monoallelic expression and its maintenance is a non-linear network, with many feedback loops and redundancies that may require rethinking of whether there can be a single necessary and sufficient causative agent. Resolving this problem is an important current question in trypanosome biology.

However monoallelic expression is ultimately achieved, it is flexible. The property of exclusive expression is frequently transferred to another *VSG* by switching, and it is to this that we now turn.

The VSG coat is switched frequently, spontaneously, and stochastically

An individual parasite will occasionally change its expressed *VSG* gene, causing the replacement of its VSG surface coat with one composed of the new variant. This

process is frequent, occurring within a lineage as often as once per 100 divisions (Turner and Barry 1989). It is spontaneous, occurring in culture (Horn and Cross 1997) and in immunodeficient animals (Magez et al. 2008). It is also stochastic: switching does not appear to be a deterministic process, and is best understood probabilistically (Lythgoe et al. 2007). Emerging from these discrete switching events is abundant antigen 'richness' across the resident trypanosome population.

The primary source of this diversity is within the genome of the infecting trypanosomes, in the form of an extensive archive of 'silent' *VSG* genes. Genes in the silent archive are found in one of two locus types in addition to silent ES: minichromosomes and *VSG* arrays. Minichromosomal *VSG* are located close to the telomeres of the short (30 to 150 kb), linear minichromosomes, which otherwise consist primarily of repeats of a 177-bp motif (Weiden et al. 1991; Wickstead et al. 2004). It is thought that most minichromosomes contain a *VSG* proximal to each telomere, giving a total of approximately 200 minichromosomal *VSG* (Barry et al. 2005; Van der Ploeg et al. 1984). Most silent *VSG*, however, are found in tandem arrays in the subtelomeres of the parasite's standard diploid ('megabase') chromosomes. The first trypanosome strain to be comprehensively sequenced has a comparatively small *VSG* archive, yet its *VSG* arrays are still thought to contain between 1000 and 2000 *VSG* altogether (Berriman et al. 2005). A large part of the archive is annotated in this strain, in the current TREU 927 genome assembly available from TriTrypDB (Aslett et al. 2010), but it is likely that a significant fraction remains missing from the current archive annotation, due to poor coverage of minichromosomes, and to the fact that in most cases only one of each pair of homologous chromosomes is represented. Figure 3a illustrates the subtelomeric arrays annotated in in the current version of the TREU 927 genome.

VSG in both locus types usually exist as cassettes with sequences at either end of the cassette that are homologous to those found in expression sites—AT-rich 70-bp repeats at the 5' end, and a conserved VSG-specific sequence at the 3' end (Figure 3b, Marcello and Barry 2007a; Pays et al. 1981; Van der Ploeg et al. 1984). A striking feature of silent *VSG* that was revealed by the genome project is that only a small proportion of the array *VSG* (4.5%) encode full-length, intact genes (Berriman et al. 2005). This small proportion is in contrast with the minichromosomal *VSG*, which are

thought to be mainly intact (Taylor and Rudenko 2006). The remainder of the array *VSG* are considered atypical due to predictions of inconsistent folding or post-translational modifications (9.5%); or are pseudogenes, either containing frameshifts or stop codons (65%), or encoding only one of the two necessary VSG domains (21%) (Berriman et al. 2005; Marcello and Barry 2007a). These non-intact genes are nonetheless utilised during infection as substrates for 'mosaic' *VSG* gene assembly, as discussed below.

There are broadly two mechanisms by which this silent archive can be accessed, and hence switching can be achieved: transcriptional switching, and recombinatorial switching. Transcriptional switching does not require any genetic rearrangement. As *T. brucei* has multiple expression sites, a different VSG will become expressed when the property of exclusive expression is transferred from one expression site to another. Transcriptional switching occurs rapidly in the bloodstream form. A simple model would involve the ESB moving to a different ES (Navarro and Gull 2001), but the triggers and mechanism of this process are vague. Furthermore, it appears that other factors are at work: the activation of a new ES and the silencing of the old one are separable processes, with chromatin remodelling required to silence the old ES (Figueiredo et al. 2008). The role of transcriptional switching in natural infections is unclear—only a small subset of *VSG* are present in ES and hence accessible *in situ*, and analyses of field-relevant trypanosome lines shows that over the course of a single infection transcriptional switching is a minor process compared with recombinatorial mechanisms (Robinson et al. 1999). Instead, it has been proposed that transcriptional switching is primarily a means of accessing different ESAG collections: the ESAGs present in different ES could evolve to the peculiarities of different host species (Bitter et al. 1998; Salmon et al. 2012; Young et al. 2008) .

Recombinatorial switching involves activation of a silent *VSG* by its copying—or complete migration—into an active expression site. This process occurs by multiple mechanisms, the most important being gene conversion. In its best-understood form, *VSG* gene conversion co-opts the ancient DNA repair mechanism of homologous recombination to delete the existing ES-occupying *VSG* and replace it with a different *VSG* from elsewhere in the genome (Morrison et al. 2009). The trigger for recombination is thought to be damage to the active ES, for example a double-

stranded break in the DNA (Boothroyd et al. 2009): a likely occurrence, given the extremely exposed nature of the DNA in the active ES and the physical instability of the AT-rich 70-bp repeats just upstream (Lin et al. 2009; Stanne and Rudenko 2010). Homologous recombination relies on similarities between DNA sequences, and rates of recombination are reduced as the length and degree of homology between substrate and template decrease (Barnes and McCulloch 2007). Sequence examination of ES which had undergone gene conversion revealed that the 5' boundary of recombination was usually located in the 70-bp repeat regions located upstream of the ES *VSG* (Liu et al. 1983), and the 3' boundary of recombination was usually in a region spanning the 3' end of the *VSG* (Bernards et al. 1981; Liu et al. 1985; Michels et al. 1983; Timmers et al. 1987). Fittingly, these regions correspond with the conserved boundaries of the '*VSG* cassette' (Figure 3c). A straightforward model of recombinatorial switching therefore sees an archival *VSG* cassette replacing the telomere-proximal *VSG*-containing section of the ES, from the 70-bp repeat region to the 3' UTR of the *VSG* or beyond (Morrison et al. 2009). An additional pathway of recombinatorial switching is available to *VSG* present at telomeres, such as those of the minichromosomes. Here, classical recombination acts to reciprocally exchange chromosome ends (Pays et al. 1985), swapping the active ES *VSG* with another telomere-proximal *VSG*. Trypanosomes deficient in the key homologous recombination enzyme RAD51 show a greatly reduced rate of switching (McCulloch and Barry 1999). Yet residual recombination events can still occur, by a process that requires only very short regions of homology, and can tolerate mismatches (Conway et al. 2002). There are clearly further mechanisms at work, occurring at lower frequency and with greater flexibility.

Recombinatorial switching does not always include the entire *VSG*, and can combine part of the archive gene with part of another. Such 'segmental gene conversion' can occur anywhere in the *VSG*, including in the antigenically important NTD, and generates a mosaic *VSG* (Kamper and Barbet 1992; Roth et al. 1989; Thon et al. 1990). Mosaic *VSG* have multiple donors, each of which can contribute multiple segments to form an expressed *VSG*: genes comprising 15 segments from four different donors have been observed (Kamper and Barbet 1992). Donors to the same mosaic *VSG* show sequence similarity to one another, but the size of contributed segments can be very small, and the long regions of near-perfect identity that are

required for classical homologous recombination in *T. brucei* do not seem to be necessary (Barnes and McCulloch 2007, J.P.J.H, Huanhuan Wang and J. David Barry, submitted). The presence of mosaic genes is not merely an inconsequential by-product of high recombination in a region with multiple homologous genes, but has two important features that could contribute to antigenic variation. Firstly, assembly of mosaic genes from fragments of pseudogenes allows accommodation of damaged *VSG*s, and access to epitopes encoded in the archive that would otherwise be unable to contribute to antigenic variation, a feature that may be crucial in the context of an archive under a hypermutation protocol (see below). Secondly, use of mosaics introduces an extra level into antigenic variation, that of recombinational variability. The construction of mosaics in each new infection allows donors to combine in multiple different ways, potentially increasing many-fold the antigenic profiles obtainable from the archive. Related *T. brucei* mosaic VSG have been shown experimentally to be antigenically distinct (J.P.J.H, Huanhuan Wang and J. David Barry, submitted), and other antigenically variant pathogens *Anaplasma spp.* and *Borrelia burgdorferi*, leverage segmental gene conversion to generate huge numbers of antigen variants (Coutte et al. 2009; Zhuang et al. 2007).

Yet mosaic *VSG* formation is also likely to be a risky process. Premature stop codons could easily be introduced into the mosaic by out-of-frame recombination events or involvement of damaged donor regions. The translated mosaic may not fold correctly, may lack key structural features such as conserved cysteines or glycosylation signals, or may be incapable of forming an effective coat. Although it is possible that as-yet-undefined cellular mechanisms exist to recognise and resolve such errors, the likelihood is that the generation of VSG by assembly of mosaics is hugely inefficient at the level of an individual trypanosome. However, at the level of the population, which can number in the hundreds of billions within a host (Barry et al. 2012), these risks can be accommodated, allowing mosaic formation to greatly enhance the potential for antigenic variation. Indeed, it appears that mosaic VSG are abundant once beyond the early stages of infection (Marcello and Barry 2007a)

Each switching process requires the convergence of different factors, and as such each has a different chance of occurring to a given silent *VSG*. The resultant variation in activation probability between silent *VSG* is the basis of an overall hierarchy of

expression across infection. Those *VSG* that are readily activated tend to be important earlier on in infection; later on, they are still likely to be frequently re-activated, but parasites that express them would be rapidly eliminated if the immune responses previously raised against their coats were still effective (Morrison et al. 2005). Other *VSG*, which perhaps require complex segmental gene conversion events to be utilized, are less likely to be activated, and so they become important later on in infection, once immune responses have appeared against the easily-activated *VSG*. ES *VSG*, resident in telomeric sites, appear to be preferentially activated early in infection. Such early activation is probably because telomeres tend to interact with one another, which promotes recombination (Barry et al. 2003), and because, compared with the archive *VSG* cassettes, the inactive ES provides more sequence that is homologous to the active ES (Hertz-Fowler et al. 2008). Intact array and minichromosomal *VSG* are usually activated somewhat later, because they have shorter stretches of more variable homology to the active ES (Liu et al. 1985; Robinson et al. 1999). However, only a single step is needed to activate an intact *VSG*, so such genes are usually expressed earlier in infection than are mosaics composed of pseudogenic *VSG* segments, which require assembly by an inefficient process of segmental conversion and hence appear later (Roth et al. 1989). The hierarchy is flexible, with previously 'late' *VSG* able to occupy more easily-activated genomic locations and thus taking an earlier position in the hierarchy (Laurent et al. 1984). The significance of and selection pressures acting on hierarchy in expression are unclear; hypotheses explaining its importance include a need to co-ordinate expression somewhat across an infection population, so as to exhaust neither archive (Morrison et al. 2005) nor host (Turner 1999). Nevertheless, hierarchies have been identified in a number of antigenic variation systems, with *Borrelia hermsii* (Barbour et al. 2006) and *Plasmodium falciparum* (Recker et al. 2011) both exhibiting clear trends in the patterns of variants that appear.

The molecular processes described in this section, summarised in Figure 2, underlie the observed patterns of frequent, spontaneous, stochastic switching, the consequence of which is vast sustained diversity amongst the trypanosome population. Calculations based on total population size in a cow and the estimated *VSG* switch rate suggest that by the first peak of parasitaemia, more than 10^8 switches will have occurred (Barry et al. 2012). Experimental studies of chronic infections of

mice have identified at least 15 distinct variants within individual samples, with many more likely to be present (J.P.J.H, Huanhuan Wang and J. David Barry, submitted).

Host immune responses shape expressed diversity

Host responses shape expressed VSG diversity, resulting in the patterns of antigenic variation. Antibodies against VSG clearly play a central role in the host adaptive response to trypanosome infection (Guirnalda et al. 2007; Magez et al. 2008), killing parasites by fixing complement, which can lyse parasites directly (at least *in vitro*, Van Meirvenne et al. 1995) or activate parasite-killing macrophages (Guirnalda et al. 2007; Pan et al. 2006). Immune responses to VSG are expected to constantly select for novelty in VSG expression, giving a directionality to the progression of antigenic variation as variants are successively neutralised (Barry and McCulloch 2001). However, trypanosome infection can have a substantial suppressive effect on host immunity (Askonas et al. 1979). *T. brucei* infections of mice can induce apoptosis in marginal zone B-cells, rendering hosts susceptible to re-challenge with previously encountered antigens, including VSG coats (Radwanska et al. 2008). It is possible that this phenomenon is unusually exaggerated in hosts such as mice that sustain exceptionally high parasitaemia for their body mass (La Greca and Magez 2011). In other hosts B-cell dysfunction and its consequences may be less extreme, manifesting perhaps in the occasional reappearance of 'early' variants in the chronic stage of infection, as such variants might not be effectively eliminated if they are re-activated. If, on the other hand, absolute B-cell dysregulation is widespread amongst natural hosts, the selection pressures favouring the evolution of the elaborate system of trypanosome antigenic variation would be mysterious, since parasites would only need to possess a handful of distinct antigens if they were able to abrogate immunological memory altogether. Investigations on a broader range of hosts are required to resolve the relative contributions of antigenic variation and immunosuppression to trypanosome persistence.

Antigenic variation interacts closely with trypanosome transmission

Alongside the extrinsic force of the immune system, trypanosomes have an intrinsic mechanism of population control (Magez et al. 2008; Seed and Sechelski 1988), linked to transmission. Trypanosomes have a complex life cycle, encompassing numerous host species, and their success relies on efficient infection of

feeding tsetse flies and efficient establishment of infection in new hosts. In fact, prolonged bloodstream presence is necessary only in so far as it enables transmission, and mechanisms that promote transmission are therefore likely to be under strong selection. Infection of a tsetse fly requires viable transmission form ('short stumpy') parasites to be taken up in the blood meal of a feeding fly (Gibson and Bailey 2003). Stumpy forms are generated by irreversible differentiation of resident proliferative form ('long slender') parasites, from which they differ in a number of ways (MacGregor et al. 2012), most notably in that the stumpy form does not replicate and has a half-life of only 48–72 hours (Turner et al. 1995). As a consequence, stumpy forms do not switch *VSG*, in fact, *VSG* transcription in stumpy forms is suppressed within the limit of detectability (Amiguet-Vercher et al. 2004). Stumpy form differentiation is triggered by an as-yet-unidentified, soluble, parasite-produced signal, 'stumpy induction factor' (SIF) (Vassella et al. 1997) which has a density-dependent effect on the population, with two consequences: providing a plentiful source of stumpy forms that maximize tsetse infectivity (MacGregor et al. 2011); and creating a negative feedback loop that stabilizes total parasitaemia, preventing early death of the host (Seed et al. 2003). Every host has a carrying capacity and the limitations imposed are predicted to have a crucial role in the dynamics of antigenic variation. For example, carrying capacity varies between hosts, with some species—those with larger blood volumes, for example—able to harbour much larger populations of parasites than others. The larger the parasite population size, the greater the chance of a particular switch event occurring, and therefore for large populations an acceleration of the kinetics of antigenic variation occurs (Barry 1986), as variants become activated sooner (Gjini et al. 2010).

Stumpy form parasites are abundant during infection (MacGregor et al. 2011). A high rate of differentiation reduces the population of parasites undergoing VSG switching to a smaller set of 'stem cell'-like slender forms, with important consequences for the dynamics of antigenic variation. If the effects of SIF are variant-independent, differentiation could maintain rarer variants at low abundance in the slender form. Given that there is likely to be a lower threshold to the size of inoculum necessary for induction of specific immunity (Morrison et al. 1982), these subpopulations could be suppressed 'below the radar' of immune sensitivity by differentiation (Gjini et al. 2010) until a dominant VSG-expressor is eliminated by an

immune response. This event opens a space in the host's carrying capacity, allowing one or more of these cryptic slender form subpopulations to expand to form part of the next wave of parasitaemia. As the expressed VSG diversity increases, relative to the sensitivity of the immune response, the greater the role of differentiation in controlling parasitaemia, since the number of different variants at low concentration cannot all induce sufficiently powerful specific immune responses for their rapid elimination. Similarly, the larger the size of the trypanosome population, the greater the likelihood that harder-to-activate variants will appear in an infection, since switching is parasite-intrinsic. Increasing the number of variants that become activated tilts the balance of infection towards differentiation-based control (Gjini et al. 2010), increasing opportunities for tsetse transmission, but also risking premature host death as a consequence of persistent high parasitaemia (Seed et al. 2003). Negotiating this trade-off may have been important in shaping the expression hierarchy (Gjini et al. 2010). In this vein it is interesting to note that both rates of VSG switching and production of transmission forms are greatly reduced in extensively syringe-passaged trypanosomes, a phenotype that can be reversed by passing through a tsetse fly (Turner 1997). Undergoing switching may impose a cost arising from risky recombination events; by artificially changing the lifecycle to one where the ability to sustain prolonged infection confers no benefit, frequent syringe-passaging or *in vitro* culture likely select for parasites that have turned down the switch rate. Whether and how the changes in lifecycle undertaken by non-tsetse transmitted trypanosomes impact on their patterns of antigenic variation would be an interesting subject for further investigation on this topic.

Antigenic variation can promote superinfection and reinfection

A trypanosome genome does not exist in isolation. Although tsetse flies have a broad host range, the high levels of infection found in wild populations (e.g. Njiokou et al. 2006) suggests that they are likely to feed on hosts which have previously been infected or are already harbouring a trypanosome infection, and thus which already are likely to have pre-circulating responses or immunological memory to many different VSG. Indeed, many tsetse flies are infected with more than one trypanosome genotype (Balmer and Caccone 2008; Macleod et al. 2001). Trypanosomes are therefore likely to encounter other trypanosome clones, both directly within an individual host, and—where destruction of immunological memory is not absolute

(Radwanska et al. 2008)—indirectly through the immunological memories of hosts. These interactions between strains are likely to be a major pressure on trypanosomes, shaping lifecycle features including antigenic variation and the *VSG* archive.

The effect of this pressure is apparent amongst VSG-expressing metacyclic trypanosomes entering a host in the bite of a tsetse fly. Establishing a successful infection in a previously infected, partially immune host requires the population of infecting parasites to evade circulating immunity, which may explain why metacyclic trypanosomes express VSG from specialized metacyclic ES (Barry et al. 1998). Each developing metacyclic trypanosome activates one of dozens of metacyclic ES, apparently at random, and so the metacyclic population shows diversity in VSG expression: as many as 27 different antibodies were required to neutralise all the metacyclic trypanosomes from a single tsetse fly (Turner et al. 1988) . Moreover, the *VSG* present in the metacyclic expression sites undergo gradual turnover (Barry et al. 1983), facilitating superinfection and reinfection of previously infected hosts.

The diversifying selection imposed by host immunity is expected to favour such strain-specificity, and indeed, this pressure extends beyond the metacyclic VSG. The stochastic formation of mosaic VSG over the course of infection could be viewed as a mechanism of generating infection-unique variants. On a broader timescale, the complement of archive *VSG* is itself likely to develop strain-specificity as the component genes evolve and diversify, as described further below.

Inter-clone competition may have acted in other ways to shape antigenic variation. Within a host, it is likely that there is competition for resources or carrying capacity, particularly if different clones use the same SIF signalling pathways (MacGregor and Matthews 2012). Under these conditions clones that frequently undertake inefficient switching events such as mosaic construction will suffer a disadvantage when competing against clones that switch only to intact genes or switch only rarely. A successful antigenic variation protocol, when in competition with others, will need to balance efficiency of switching with scope of variability as an infection progresses. The battery of easily accessible minichromosomal *VSG* deployed early in infection may represent a compromise, allowing risky mosaic *VSG* construction to be relegated to the chronic stage of infection. Antigenic variation is not just a battle between a

parasite and its host, and the effects of inter-strain competition on the diversity phenotype represents an interesting subject for further study.

The VSG *archive is adapted to promote rapid change of the repertoire*

Given that uniqueness and diversity in the *VSG* archive are likely to be favoured, the trypanosome genome itself is in a constant state of flux. While the 'trypanosome genome' is an extremely useful resource (Berriman et al. 2005), it represents only a snapshot of the genome at a single point in time. The genomes of kinetoplastids are extremely plastic: for example *Leishmania* species display considerable variation in chromosome and gene copy number (Rogers et al. 2011); and the sizes of homologous chromosomes can vary considerably between *T. brucei* strains (Melville et al. 2000). In trypanosomes, this variability has been co-opted in the evolution of the *VSG* archive. Diversity within the archive is ancient, as evidenced by the fact that multiple contemporary *VSG* lineages are shared between African trypanosome species (Jackson et al. 2012). However, the archive continues to evolve rapidly, such that differences in *VSG* archives may account for most of the difference in chromosome size between strains. For example, in one examined strain, one copy of chromosome 1 has over half of its length devoted to *VSG*— around 3 Mb, potentially 600 *VSG* —but in the genome strain there are fewer than 10 *VSG* currently annotated in the haploid chromosome 1 assembly (Berriman et al. 2005; Callejas et al. 2006; Marcello and Barry 2007a).

Genome variability is apparent not only in the size of the archive but also in the sequence of *VSG* genes comprising it. Strains that have minor differences in housekeeping genes can have large differences in their *VSG* repertoires, and repertoires appear to have diverged to become strain-specific (Bernards et al. 1986; Hutchinson et al. 2007). However, it is not immediately obvious how selection could promote these changes in the genome. An individual *VSG* gene will be invisible to selection unless it is expressed, which most are not; and the size of the archive means there is a degree of redundancy: any individual gene can be lost with probably little effect. It therefore seems unlikely that the rapid rate of change is generally the result of strong selection on individual *VSG* genes. More likely is the hypothesis that 'second-order selection' (Caporale 2003) for diversity in expressed VSG has

promoted the evolution of *mechanisms* that generate mutations of various sorts in *VSG* genes, resulting in the observed hyperevolution (Barry et al. 2012).

An important adaptation promoting the evolution of diversity in *VSG* genes is their location in subtelomeres. Subtelomeres are the transitionary regions between the low-complexity telomere repeats at the tips of linear chromosomes, and core regions, in which gene content, order and intergenic sequence are shared between homologous chromosome partners. A key feature of subtelomeres is their rapid rate of change compared with chromosome cores (Riethman et al. 2005). Elevated rates of ectopic and homologous recombination in the subtelomeres promote diversity by allowing subtelomeric genes to exchange sequence in a process of segmental conversion; and by duplicating genes (Linardopoulou et al. 2005; Mefford and Trask 2002). Gene duplication may lead to relaxation of selection on one of the copies and open the possibility of neofunctionalisation, as has been studied in subtelomeric families of disaccharide utilisation genes in yeast (Brown et al. 2010).

Most subtelomere sequence is non-coding, but these regions are frequently home to members of large, highly diverse families. *T. brucei* is only one example of a pathogen that has located in subtelomeres gene families that are important to a diversity phenotype: other subtelomeric gene families include the *var*, *rif* and *stevor* antigenic variation gene families of *P. falciparum* (Duffy and Tham 2007; Gardner et al. 2002; Hernandez-Rivas et al. 1997; Kyes et al. 1999; Scherf et al. 2008); the *vir* superfamily of *Plasmodium vivax* (Fernandez-Becerra et al. 2009); the major surface glycoproteins used in antigenic variation *Pneumocystis carinii* (Stringer and Keely 2001); and various surface protein genes in *T. cruzi* (Moraes Barros et al. 2012).

Partitioning of *VSG* genes in a different environment from chromosome cores provides a means for *VSG* to be subject to different mutational activities from core genes, for which a high mutation rate could be catastrophic. Specifically, the location of *VSG* in the subtelomeres means that these genes are exposed to the hypermutational environment of these regions, which influences the evolution of *VSG* in several ways (Barry et al. 2003). Firstly, the high subtelomeric recombination rate will likely promote duplication of *VSG*. The duplication of intact genes might be a mechanism for replenishing the archive, compensating for the degeneration of pseudogenes and their eventual loss of from the usable archive (Nei and Rooney

2005). Other mutagenic processes acting on the two gene copies could then eventually produce two antigenically distinct VSG. The frequent occurrence of duplications also has an effect beyond creating more *VSG* genes, because it results in the presence of subfamilies in the archive: *VSG* genes have very low identity to most others in the archive, but around 40% of genes in the TREU 927 archive have a high-identity partner, presumably due to a recent duplication (Marcello and Barry 2007a). This subfamily structure is thought to be key to providing multiple donors of sufficient identity to each other to assemble mosaic *VSG* genes, allowing evolving diversity in pseudogenes to be accessed. The existence of closely-related genes within the archive, combined with the high level of ectopic recombination in the subtelomere, can also generate new combinations of *VSG* sequence through a process of segmental conversion (Gjini et al. 2012b). There is some debate as to whether recombination is capable of increasing diversity (Martinsohn et al. 1999), but it is possible that an effect of ectopic recombination in *VSG* arrays may be to assemble antigenically novel genes in a process analogous to the generation of mosaic VSG during infection. Interestingly, it appears that recombination between *VSG* has been less important in shaping the *T. congolense* and *T. vivax* archives, suggesting that antigenic diversity is generated by distinct mechanisms in each species (Jackson et al. 2012).

Secondly, comparison of genes within the TREU 927 *VSG* archive has been used to infer the events of *VSG* evolution, and these analyses have suggested that smaller-scale mutational processes are also important, namely point mutation and short insertion-deletions (Gjini et al. 2012b; Marcello and Barry 2007a). Analysis in our laboratory of the *VSG* archives of sequential isolates of one trypanosome strain has indicated that substitution mutation processes are both qualitatively and quantitatively different in *VSG* from cores, with substitution mutations accumulating at silent sites at a rate several times higher in *VSG* than in core genes (L.P., T. Otto, M. Berriman, and J.D. Barry, with permission). The role that such small mutations might play in antigenic variation is unclear. The scale and rate of point mutations indicate that point mutation is unlikely to produce antigenically novel sequence rapidly enough to make a substantial contribution to antigenic variation in a single infection of a single host (Graham and Barry 1996). However, the accumulation of small mutations over a longer timescale could introduce new diversity to the archive, and might give an

advantage at the scale of the parasite population if it allowed infection of a previously infected host.

As noted above, *VSG* archives vary considerably between strains. The occurrence of changes at the sequence level are a key feature of *VSG* evolution, but other factors are also thought to be involved in building archive strain-specificity. One hypothesis is that trypanosomes have ceased to exchange sequence between telomeres and subtelomeres during meiosis. Meiotic recombination tends to homogenise gene sequences between homologous chromosomes, and the removal of this homogenising effect would promote the divergence of archives between strains as they accumulated different changes (Hutchinson et al. 2007). A second possible factor is the operation of population bottlenecks: migration to the tsetse salivary glands appears to be a severe bottleneck in the parasite life cycle (Oberle et al. 2010; Van Den Abbeele et al. 1999), and as such could rapidly fix different variants in different populations.

The subtelomeric *VSG* arrays, therefore, represent a large repository of information available for the trypanosome to use in antigenic variation, but their role goes considerably beyond that of an inanimate archive. Rather, the arrays are potent generators of diversity, driven by an active hypermutation protocol. The mutations that occur under this protocol are likely to introduce pseudogenicity, which imposes a requirement for the formation of mosaic *VSG* during infection, if the full range of antigens is to be exploited. Additionally, the accumulation of mutations contributes to strain specificity. Thus, the structure and evolution of the *VSG* archive plays a key role in antigenic variation at several levels (Gjini et al. 2012a).

Going forward

In summary, African trypanosome antigenic variation is a genome strategy for prolonged infection and transmission, a diversity phenotype that emerges at the population level amongst clonally related parasites. The antigenic variation system operates through an immunogenic surface coat of VSG, which shields invariant surface molecules and protects the cell from the innate immune system. Expression can readily switch to a different VSG resulting in a diverse population that pre-empts adaptive immunity. The diversity phenotype emerges at the population level from the operation of a complex network of molecular actors that maintain strict monoallelic

expression and effect switching between VSG in a frequent, spontaneous and stochastic manner. The *VSG* repertoire, although containing a huge number of silent *VSG*, contains many pseudogenes. The construction of mosaic *VSG* allows use of these pseudogenes and may also be important for introducing combinatorial diversity into the system and allowing the generation of infection-specific variants. In the longer term, the evolution of diversity is promoted by adaptations that shape the *VSG* archive, notably the location of the main part of the archive in hypermutational subtelomeres. Antigenic variation is likely to have a profound influence in the dynamics of infection and superinfection, which are determined by the interaction of many factors including the probabilistic order of VSG expression, differentiation, the action of immune effectors, and inter-strain competition.

African trypanosome antigenic variation represents a highly successful survival strategy, and as such is an inherently interesting biological phenomenon. Currently, a considerable amount of detail is being elucidated in terms of understanding the molecular mechanisms of antigenic variation, with demonstration of involvement for numerous proteins (e.g. reviewed in Alsford et al. 2012 and Morrison et al. 2009). In the future, perhaps we can look forward to the integration of these data into a more complete model of the mechanisms of antigenic variation from the molecular level upwards. Important to the development of any model would be a consideration of low frequency events which are likely to occur in the large populations in which antigenic variation is made manifest. For example, elucidating the molecular details and temporal dynamics of mosaic VSG assembly would provide a key to the processes facilitating chronic infection. Further illumination would be given by a better understanding of the infection biology and antigenic variation systems of non-*brucei* African trypanosomes, which are beginning to be less neglected (e.g. Chamond et al. 2010; Coustou et al. 2010; Greif et al. 2013). Such comparative studies, along with the wider availability of genome data, are also yielding insights into the evolution of the *VSG* archive (Jackson et al. 2012); further research on these patterns will be useful in inferring the selective pressures that have shaped this system in related species. Increasing availability and sensitivity of deep sequencing techniques will be invaluable in dissecting the composition of the trypanosome population over the course of infection, which could become a powerful tool in the study of infection dynamics. Finally, it will be important to resolve the within-host interaction, and the

broader evolutionary interplay, between antigenic variation and immune suppression, as the details and significance of the latter become better understood. Because it is so closely allied to various fundamental aspects of parasite biology and dynamics of population diversity, future studies of trypanosome antigenic variation are likely to provide insights not only in understanding this specific system, but also into broader questions associated with fields as broad-ranging as gene expression, DNA recombination, host-parasite co-evolution and biological diversity.

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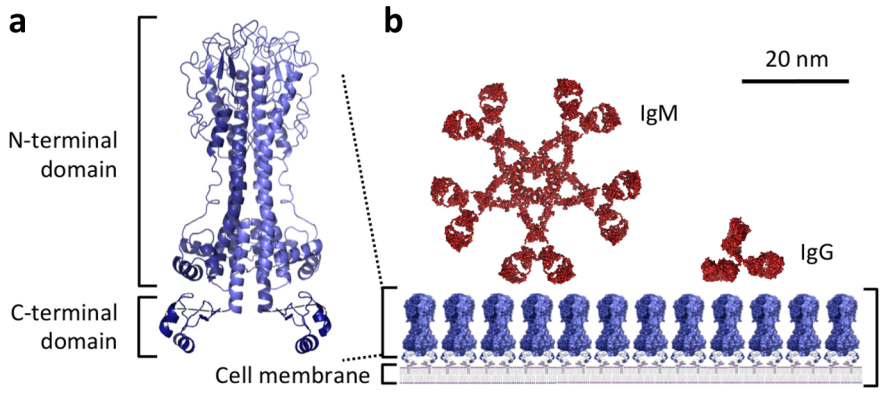

Fig. 1 a Each VSG dimer is an extended structure consisting of an N-terminal domain (NTD) and a C-terminal domain consisting of one or two subdomains. The membrane-distal end of the NTD is the region of the glycoprotein exposed to immune effectors on an assembled surface coat. **b** The assembled surface coat sterically hinders access of host immune effectors to the cell membrane or invariant surface proteins. Images were assembled using Protein Data Bank structures 1vsg, 1xu6, 1rcj and 1igt, were visualized using Pymol (Schrödinger, LLC), and were inspired by Engstler et al. (2007).

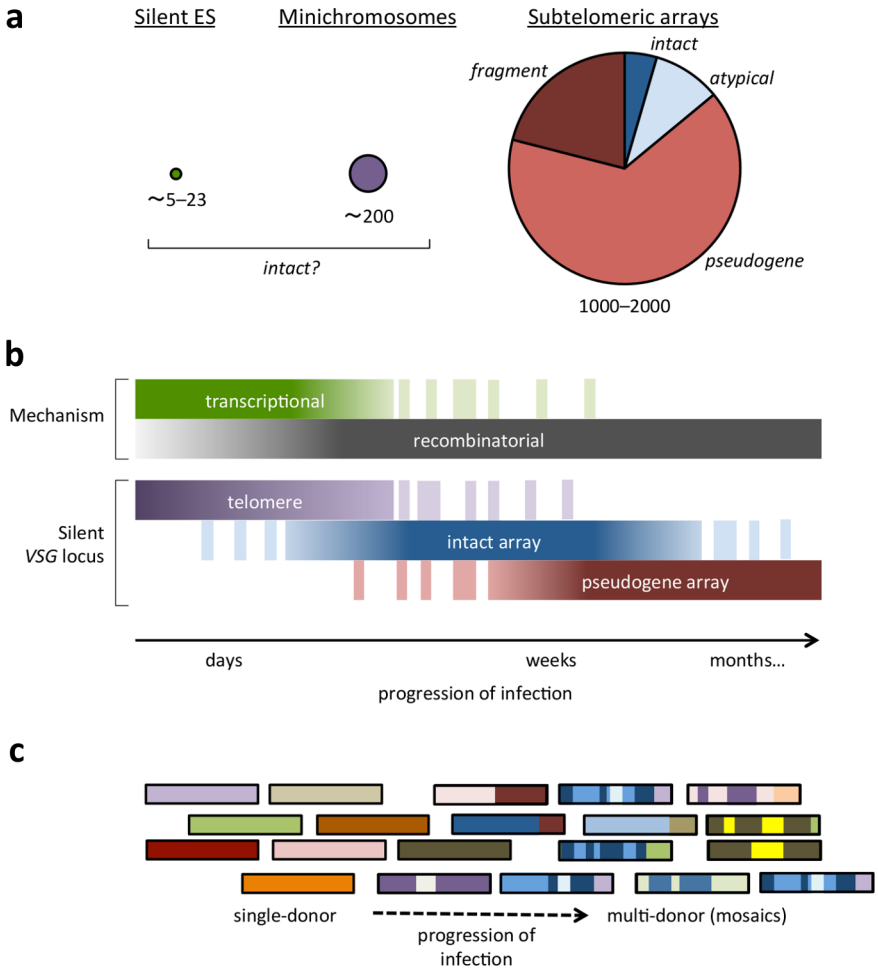

Fig. 2 a Several different locus types contain the silent *VSG* archive. Circles indicate archive *VSG* present at silent ES, minichromosomes and in subtelomeric arrays. The areas of the circles are approximately proportional to the relative number of *VSG* at each location type. The approximate number of *VSG* present at each location is given below each circle; the exact number varies between strains. In the case of the subtelomeric arrays, the circle is divided proportionally according to the intactness of silent *VSG* (after Marcello et al., 2007). Silent ES and minichromosomal *VSG* are thought to be largely intact. **b** *VSG* are expressed according to a hierarchy. For each process, intensity of shading indicates the relative importance in activating novel *VSG* over the course of infection. Recombinatorial switching assumes prominence over transcriptional switching beyond the early stages of infection. Telomere-resident *VSG*

in silent ES and minichromosomes are activated more readily than those in the *VSG* arrays. Pseudogenic *VSG* require low-probability segmental gene conversion events to be accessed, and tend not to appear until the chronic stage of infection in the form of mosaic *VSG*. This diagram was inspired by Morrison et al., (2009). **c** Expressed VSG are diverse, and mosaic *VSG* become increasingly predominant as infections progress and immunity neutralises readily-activated single-donor VSG. Coloured rectangles are a stylised representation of expressed *VSG* that might be sampled at different points of infection. Different colours represent different donors. Note that it is likely that the total population of expressed *VSG* is much richer at any given point than indicated here.

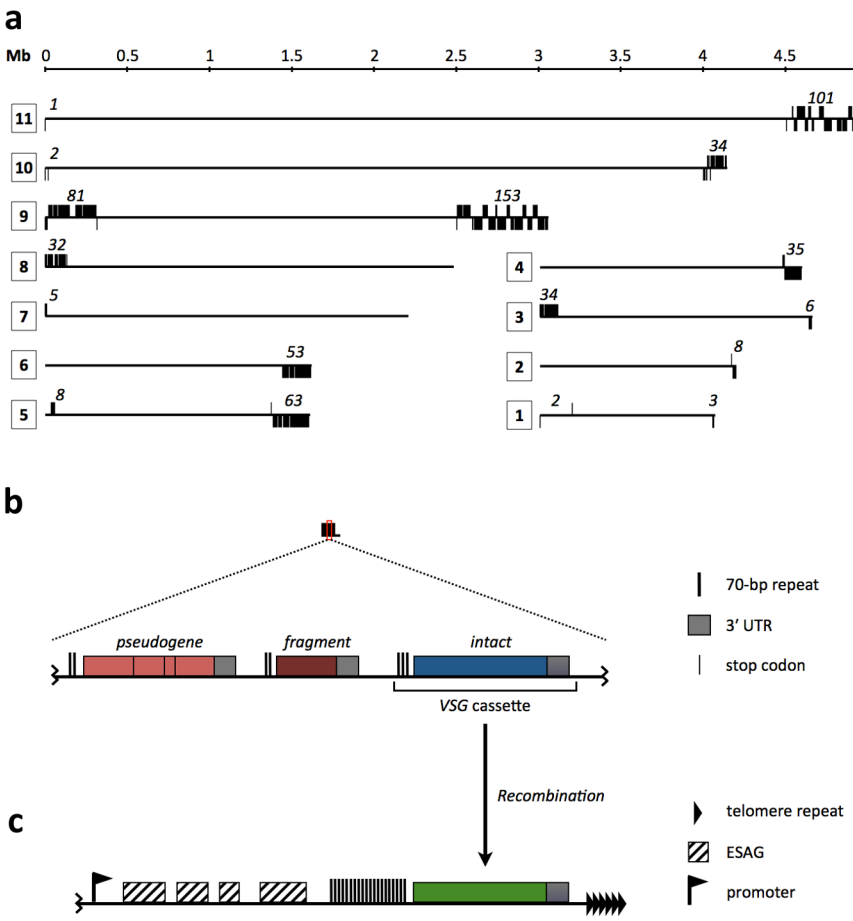

Fig. 3 a Subtelomeric *VSG* arrays are found across the *T. brucei* genome. Chromosome numbers are shown in boxes, numbers in italics above subtelomeric arrays indicate the number of genes in each in the current version of the TREU 927 genome assembly. Lines projecting above the line of the chromosome represent VSG genes, pseudogenes or gene fragments on the forward strand; those projecting below represent the same on the reverse strand. For chromosome 11, the assembly consists of a main contiguation and two much smaller fragments: only the main contiguation is shown, although the fragments not shown also contain VSG. **b** Stylised structure of part of a *VSG* array, showing *VSG* cassettes with features with homology with expression sites. The red box indicates the approximate scale in **a** of the part of the

array shown. **c** Stylised representation of the principal features of a telomeric VSG expression site.