



Emergence and adaptation of the cellular machinery directing antigenic variation in the African trypanosome

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Survival of the African trypanosome within its mammalian hosts, and hence transmission between hosts, relies upon antigenic variation, where stochastic changes in the composition of their protective variant-surface glycoprotein (VSG) coat thwart effective removal of the pathogen by adaptive immunity. Antigenic variation has evolved remarkable mechanistic complexity in *Trypanosoma brucei*, with switching of the VSG coat executed by either transcriptional or recombination reactions. In the former, a single *T. brucei* cell selectively transcribes one telomeric VSG transcription site, termed the expression site (ES), from a pool of around 15. Silencing of the active ES and activation of one previously silent ES can lead to a co-ordinated VSG coat switch. Outside the ESs, the *T. brucei* genome contains an enormous archive of silent VSG genes and pseudogenes, which can be recombined into the ES to execute a coat switch. Most such recombination involves gene conversion, including copying of a complete VSG and more complex reactions where novel 'mosaic' VSGs are formed as patchworks of sequences from several silent (pseudo)genes. Understanding of the cellular machinery that directs transcriptional and recombination VSG switching is growing rapidly and the emerging picture is of the use of proteins, complexes and pathways that are not limited to trypanosomes, but are shared across the wider grouping of kinetoplastids and beyond, suggesting co-option of widely used, core cellular reactions. We will review what is known about the machinery of antigenic variation and discuss if there remains the possibility of trypanosome adaptations, or even trypanosome-specific machineries, that might offer opportunities to impair this crucial parasite-survival process.

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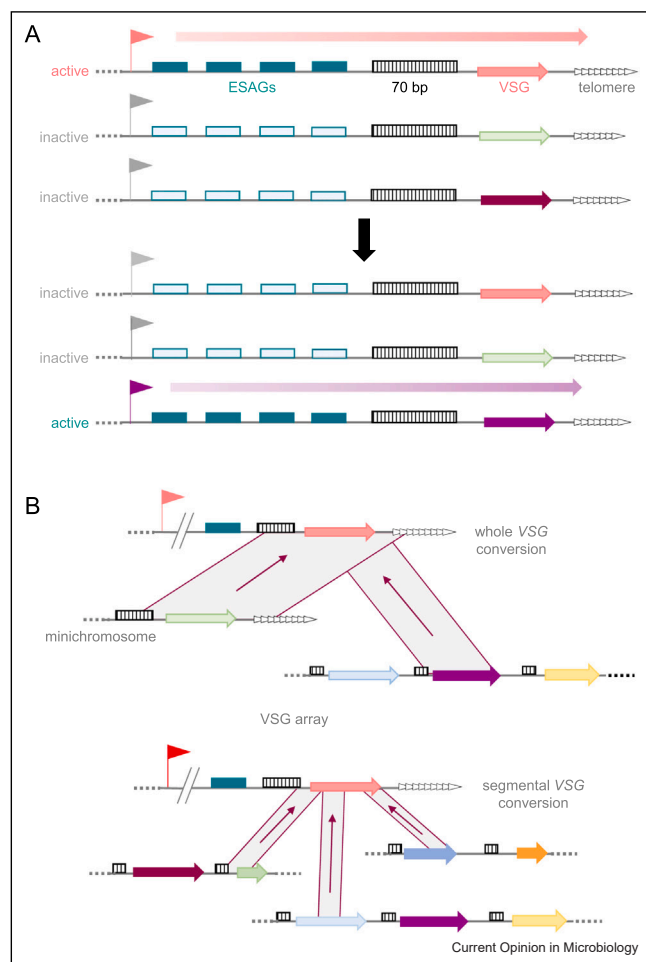
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Introduction

All pathogens of mammals, including humans, must survive innate and adaptive immune attack to prosper and transmit between hosts. Antigenic variation is a widespread strategy for immune evasion that evolved independently in bacteria, fungi and protozoans, and involves repeated, stochastic changes in exposed pathogen antigens [1]. The adaptive immune system needs time to raise specific effector immunity against currently expressed antigens, providing a window of time during which pathogens can switch to a new antigen, allowing part of the population to survive immune elimination and seed outgrowth of antigenically distinct pathogens (until the next wave of specific immune attack).

Trypanosoma brucei relies on switching its variant-surface glycoprotein (VSG) 'coat' to evade adaptive immunity, using two mechanisms. First, *T. brucei* contains ~15 telomeric expression sites (ESs, [Figure 1a](#)), which direct VSG gene transcription. Only one ES is fully transcribed in a single cell at a time, but transcriptional switches occur when the active VSG ES is silenced and a previously silent ES, harbouring a distinct VSG, is activated. Second, *T. brucei* contains thousands of silent VSG genes and pseudogenes, located outside the ESs in subtelomeric arrays in the 11 'megabase' chromosomes and at the ends of 50–100

Figure 1



Two pathways for VSG gene-expression switching during antigenic variation in *Trypanosoma brucei* antigenic variation. **(a)** One VSG (salmon arrow) is expressed at a time in a single cell, due to monogenic transcription of only one VSG ES (active) amongst ~15 found in the genome (only three silent ES are diagrammed, inactive). In each ES, a VSG is found proximal to the telomere and is separated from upstream, co-transcribed ES associated genes (ESAGs) by 70-bp repeats. The promoter (flag)-directing transcription of the ES recruits Pol-I. A switch in VSG expression can occur when the active VSG ES is silenced and one inactive ES becomes transcribed. **(b)** Recombination can activate silent VSGs from anywhere in the genome, including from telomeric minichromosomes or subtelomeric VSG arrays. The upper diagram depicts the extent of VSG and flanking sequence transferred during gene conversion of an intact VSG; note, the two events shown do not occur simultaneously. The lower diagram depicts gene conversion of multiple segments of silent (pseudo) VSGs, generating a novel mosaic VSG, to be expressed. The VSG must reside in the active ES, but whether the mosaic is constructed there (as shown) or elsewhere is unclear.

so-called minichromosomes (Figure 1b). Silent VSG (pseudo)genes can be activated by recombination reactions that move them to the ES, displacing the resident VSG.

Though there appears to be little need for new therapies against human African trypanosomiasis, given recent

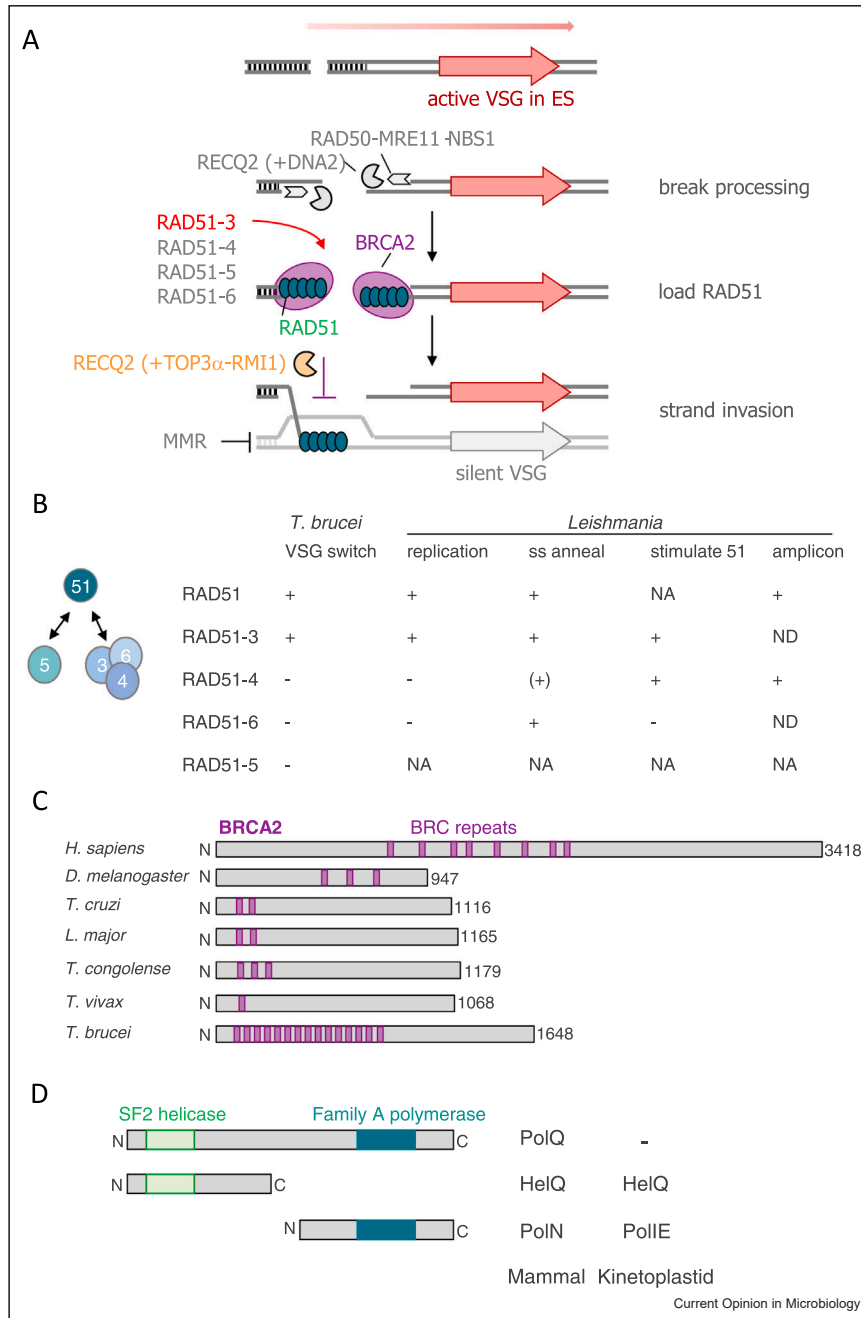
dramatic successes in controlling case numbers [2], impeding antigenic variation would theoretically provide a highly effective strategy to tackle disease causing any pathogen that uses this form of immune evasion, since sustaining and transmitting an infection would be curtailed. In *T. brucei*, loss of VSG expression is lethal even in culture [3] and, moreover, simultaneous expression of multiple VSGs impairs infection by the parasite [4]. Understanding of the mechanisms that drive antigen switching in *T. brucei* is arguably the most advanced of any pathogen, as this knowledge has accrued through decades of investigation [5–7]. Hence, lessons learned in *T. brucei* might be leveraged for other pathogens, including related animal trypanosomes, which continue to cause devastating levels of disease. However, no route to obstruct VSG switching has yet emerged, despite the wealth of mechanistic knowledge. Here, we review what is known about the machinery that directs *T. brucei* antigenic variation and discuss the difficulties of identifying a strategy to impair VSG switching when the reactions involved exploit, and are embedded within, widely conserved cellular processes for gene expression and genome maintenance.

Catalysis of variant-surface glycoprotein switching by recombination

Describing the machinery directing VSG recombination has relied on mutation of core genome-repair enzymes. Loss of Rad51, the central enzyme of eukaryotic homologous recombination (HR) [8], significantly impairs antigenic variation [9] (Figure 2a). The same effect is seen following loss of BRAC2 [10,11] or the Rad51 paralogue RAD51–3 [12,13], each of which modulates the activity of Rad51 during DNA double-strand break (DSB) repair (Figure 2a–c). In contrast, mutation of Ku, which normally directs an alternative DSB-repair pathway called non-homologous end-joining (NHEJ), has no effect on VSG switching, but alters telomere homeostasis [14,15]. These data suggest that VSG switching does not involve a novel or dedicated recombination pathway but has instead co-opted a universally conserved DNA-repair activity, a finding with considerable parallels to antigenic variation in the bacterium *Neisseria* [16,17]. Such a conclusion limits options for specific inhibition of trypanosome HR. For instance, though inhibitors of Rad51 [18,19] developed for cancer treatment may also target *T. brucei* RAD51, given the high level of sequence conservation [13,20,21], loss of HR has severe consequences for mammalian cells [22]. Nonetheless, novelties in *T. brucei* HR proteins have emerged.

T. brucei BRCA2 has evolved the largest yet described array of BRC repeats, which mediate one form of BRCA2–Rad51 interaction (Figure 2c) [10]. Intriguingly, a similar BRC expansion is not seen in *T. brucei*'s close

Figure 2



Factors implicated in VSG gene conversion in *Trypanosoma brucei*. **(a)** Factors shown in colour have been demonstrated to influence VSG recombination based on analysis of mutants, while loss of those shown in grey was found not to affect VSG switching. Available data are consistent with VSG recombination following the known steps of HR repair of a DNA DSB, with the exception of the uncertainty in how the putative break is processed by end resection. Only early steps, up to RAD51-directed strand exchange, are shown, and the factors diagrammed are discussed in the text. **(b)** RAD51 paralogues modulate the activity of RAD51 and the diagram and table illustrate the disconnect between current understanding of the complexes formed by the four RAD51 paralogues in *T. brucei*, and the non-overlapping roles played by the proteins in both *T. brucei* and *Leishmania* (ss *anneal* denotes in vitro activity in annealing single-stranded DNA; *stimulate 51* denotes promoting RAD51-directed activity in vitro; *amplicon* denotes a role in the generation or maintenance of extrachromosomal DNA; *ND* and *NA* denote not determined and not applicable, respectively). **(c)** A comparison of the size (amino acid residues) and number of BRC repeats predicted in a range of eukaryote BRCA2 orthologues. **(d)** A representation of the domain organisation of putative DNA polymerase theta (PolQ)-related proteins in mammals and kinetoplastids.

relatives, though whether this is an adaptation that serves the needs of *VSG* switching in *T. brucei* is unclear [11]. The BRC repeats of *T. brucei* BRCA2 may also mediate interactions with RAD51 paralogues, and inhibiting such interactions increases DNA-damage sensitivity and impairs growth [23]. Finally, structural work revealed that BRC–Rad51 interaction differs between *Leishmania* and mammals [24•], with implications for how BRCA2 modulates RAD51 function during HR [25]. Altogether, these observations may raise hopes for specific inhibition, perhaps by modifying compounds that impede BRC–Rad51 interaction [26].

Rad51 paralogue functions remain incompletely understood in any eukaryote [8,27], since they are a diverse, rapidly evolving family of proteins that form several complexes, providing roles in Rad51 strand exchange, DNA replication-fork processing [28] and DNA lesion tolerance [29,30]. In *T. brucei*, loss of RAD51–3 has a greater impact on *VSG* switching than any of the three other RAD51 paralogues, perhaps suggesting specific DNA-repair activities for antigenic variation. Though available data suggest three of the RAD51 paralogues interact in both *T. brucei* and *Leishmania* (Figure 2b), genetic data reveal non-overlapping functions in DNA repair and replication [13,31,32••]. Deeper analyses of these factors may shed light on the intersections between *VSG* recombination, cell-cycle progression and DNA replication, including what initiates the process (see below), and whether the conserved HR machinery for antigenic variation associates with other (perhaps novel) repair pathways.

The mystery of variant-surface glycoprotein pseudogene switching

The contribution of *VSG* pseudogenes to the formation of novel ‘mosaic’ *VSGs*, which are distinct from any single (pseudogene) in the silent archive, has long been known [33–35] (Figure 1b). The scale and complexity of the segmental gene-conversion reactions [36–40] that give rise to patchwork, functional mosaic *VSGs* have recently become clear. How — and indeed where in the genome — mosaic *VSG* recombination is catalysed is unknown; though a repair reaction termed microhomology-mediated end-joining (MMEJ) has been suggested to contribute [41], no test has been done.

In all kinetoplastids tested so far [42–47], no evidence has emerged for the use of Ku-directed NHEJ [48•]. Instead, untemplated DNA DSB repair relies on MMEJ or single-strand annealing (SSA, relying on longer stretches of homology than the imperfect ~5–20-bp homology used in MMEJ). MMEJ in metazoans involves DNA Polymerase theta (PolQ), a dual functional enzyme with both helicase and polymerase activity [49–51] that acts during DSB repair, collapsed replication-fork recovery [52•] and genome

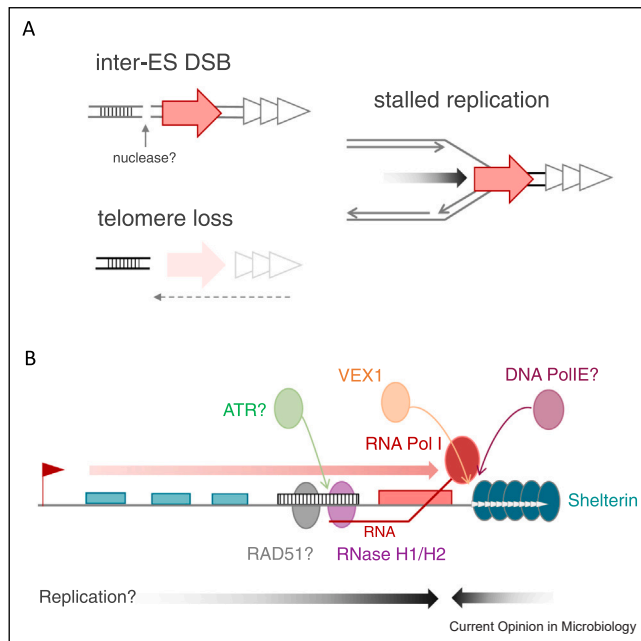
instability [53–55]. Some metazoans additionally encode stand-alone helicase (HelQ in mammals) and DNA Pol (PolN in mammals) factors (Figure 2d). Intriguingly, kinetoplastids do not encode a ‘true’ PolQ, but only HelQ- and PolN-like proteins (Figure 2d) whose evolutionary origins remain unclear [56,57••], since the latter factor, recently named PolIE [57••–59••], shares closest homology not with PolQ or PolN, but a family of mitochondrial DNA Pol-I-related enzymes [46••,57••,60]. Loss of PolIE in *T. brucei* leads to increased DNA damage and *VSG* switching (at least some by recombination) [57••] and perturbs telomere homeostasis [59••], revealing nuclear, *VSG*-associated roles. No study has explored the origins of the putative kinetoplastid PolQ factor, but its mutation in *Leishmania* impairs both MMEJ and SSA repair of CRISPR–Cas9-generated DSBs [46••], as seen in *Caenorhabditis elegans* HELQ-1 mutants [61]. Though these data do not provide evidence of functional links between HELQ and PolIE, they suggest each plays roles in genome maintenance, perhaps providing a foundation for asking if HELQ and/or PolIE might act in *VSG* mosaic gene conversion, including whether inhibitors directed against human PolQ might be active [62]. However, caution is required: yeast do not encode PolQ, HelQ or PolN but catalyse MMEJ using two unrelated DNA Pols [63]; thus, this repair mechanism may be enzymatically flexible.

Initiation of variant-surface glycoprotein-switch recombination

Recently, attention has turned to asking how the process of *VSG*-switch recombination may initiate, since the reaction can occur at rates exceeding the level of background mutation [64]. No single accepted initiation model exists, but instead three suggestions are being explored (Figure 3a) [65].

One proposed initiation model is that *T. brucei* actively generates DSBs within the *VSG* ES (Figure 3a), precipitating HR [66–68], potentially via a dedicated endonuclease that recognises a sequence or structure within the *VSG* ES [66]. There is no doubt that DSBs can elicit *VSG* switching, since expression of the yeast endonuclease I-SceI and targeting its activity to upstream of the *VSG* in the active ES elevates recombination levels [69,70]. Moreover, ligation-mediated polymerase chain reaction (PCR) can detect DNA breaks around the telomere-proximal *VSG* in both active and silent *VSG* ESs [69,71]. DSB location appears important, since DSBs targeted within the 70-bp repeats [72••], or adjacent to the promoter or telomere [71], do not elicit a switch. I-SceI-generated DSBs at the *VSG* in the active ES are typically very detrimental to *T. brucei* survival [44,70,71,73], perhaps due to perturbation of cell-cycle progression [70], and perhaps because their incomplete repair does not prevent genome replication and mitosis [74•].

Figure 3



Models and activities predicted to direct VSG switching in *Trypanosoma brucei*. **(a)** Three DNA lesions predicted to form within the active VSG ES and to precipitate VSG recombination (for simplicity, only the telomere, VSG and upstream 70-bp repeats are diagrammed). **(b)** A speculative diagram listing the range of factors implicated in VSG switching to date, and the potential locations and functions they may provide.

Current data suggest an incomplete understanding of the pathway(s) that process VSG ES DSBs to elicit switching. During HR, strand exchange requires that DSBs undergo short- and then long-range nuclease resection, the former catalysed by Mre11–Rad50–Nbs1 (Xrs2 in yeast, MRN) and the latter by Dna2–RecQ helicase and/or ExoI. Unexpectedly, mutation of *T. brucei* MRE11 does not alter VSG switching efficiency (Figure 2a), despite leading to genome instability [75]; furthermore, loss of either MRE11 or RAD50 results in increased use of the VSG repertoire after targeting an ISce-I DSB to the VSG ES [76••]. Similarly, while mutation of RECQ2 impairs survival of *T. brucei* after an ISce-I-induced DSB, including in the VSG ES, VSG-switching frequency in the absence of an induced DSB increases [73], suggesting its major role in VSG switching is not linked to VSG ES DSB processing but acting in conjunction with Topoisomerase 3a [77,78] to limit chromosome crossover and/or RAD51 strand exchange during VSG HR [79–81]. Clearly, clarification is needed regarding how DSBs are processed, signalled and repaired in *T. brucei*, including the roles of the paradoxical Ku complex, which is expressed but appears not to act in NHEJ [48•], and of the DNA-repair kinase Ataxia-telangiectasia Mutated (ATM) [82], a key orchestrator of HR DSB repair.

A second model suggests initiating lesions may be single-ended breaks resulting from telomere loss (Figure 3a) [83]. Such a scenario might explain why ligation-mediated PCR does not detect breaks at a single discrete location, reflecting randomness in the extent of (sub) telomere loss [69,71,84–86]. Indeed, increased VSG switching occurs when telomeres are disrupted by telomerase mutation [87–89], or mutation of the shelterin complex and associated factors [84–86]. Endonuclease excision of the telomere tract does not elicit switching [71,90], however, suggesting any telomere-derived break must extend into the VSG ES to affect coat change. This model can only explain recombination-directed activation of silent telomeric VSGs (within the silent ES and minichromosomes) [68], and relies on ‘one-ended’ break homology and strand exchange, perhaps more similar to break-induced replication than gene conversion [68,77]. These predictions could be tested, since data from other eukaryotes have revealed how the machinery and repair profile of break-induced replication differs from two-ended gene conversion [91,92].

A final model for VSG-switch initiation suggests that DNA breaks may not form directly but result from other lesions, such as a stalled replication fork (Figure 3a). This model is the accumulation of several strands of evidence. First, the actively transcribed VSG ES is replicated notably earlier in S-phase than all silent ES, perhaps enhancing collisions with transcription machinery [16,73]. Second, loss of two different RNase H factors in *T. brucei* leads to accumulation of RNA–DNA hybrids (R-loops) across the VSG ESs and increased VSG switching, both associated with increased DNA damage [93••–95]. If and how R-loops relate to VSG ES transcription and replication is unknown, including whether they cause or result from putative collisions, moreover, the relationship between intra-ES R-loops and telomeric RNA–DNA hybrids is still to be fully explored [86,96]. Finally, loss of the DNA-repair kinase ATM–Rad3-related (ATR) also causes increased VSG ES damage and increased switching [97••]. In other eukaryotes, ATR plays important roles in signalling replication-associated lesions, including transcription–replication clashes and associated R-loops [98,99•], but the precise mode of action of *T. brucei* ATR in the interplay between early replication of the actively transcribed VSG ES, R-loops and damage is yet to be resolved. Inhibitors of human ATR and ATM, which appear active against the orthologous kinases in kinetoplastids [100–102], could be used to explore these questions further.

A fine-tuned system for variant-surface glycoprotein monogenic expression

Unusually, *T. brucei* transcribes all ESs using RNA-polymerase-I (Pol-I). Transcription appears to initiate on all ESs, but elongation and mRNA maturation is

restricted to the active locus [103,104]. Nuclear architecture influences gene expression in multiple eukaryotes, for instance, by compartmentalising processes such as transcription and RNA processing. Similarly, chromatin condensation and nuclear arrangement of the ESs playing critical roles in sustaining VSG monogenic expression and efficient RNA processing [105••,106••]. Notably, some factors linked to these processes appear unique to kinetoplastids.

Silent VSG ESs are located at more peripheral locations in the nucleus [107,108] than the active VSG ES, which is transcribed within a central 'expression-site body' (ESB). The ESB is usually positioned adjacent to the nucleolus and contains a local reservoir of Pol-I (Figure 4) [109]. In insect-stage cells, all VSG ESs localise to the nuclear envelope and appear to form constitutive heterochromatin [108]. Several widely conserved factors regulate the repressive state of the VSG ESs, including, but not limited to, ISWI, RAP1, histone deacetylase 3, the histone trimethyltransferase DOT1B and histone H1 [110–114]. Dynamic ES positioning additionally suggests the nuclear lamina (formed mainly of NUP-1 and -2 [115,116]) may present a repressive compartment to silence ESs. Although NUP-1 disruption leads to a modest misregulation of VSGs [117], identification of other NUP-1/-2-interacting factors may identify the structural basis of heterochromatin formation critical for ES silencing.

All VSG ESs are found adjacent to telomeres, which form heterochromatic structures to prevent promiscuous DNA repair. Loss of telomeric factors can not only influence VSG recombination, but transcriptional control. For instance, knockdown of RAP1, a telomere-binding factor required for telomere stability and silencing, derepresses silent VSG ESs in bloodstream-form cells and also in insect-stage cells, where VSG expression is developmentally shut down [112]. Recently, such a silencing role for RAP1 has been found to involve an interplay with the inositol phosphate pathway, via phosphatidylinositol 5-phosphatase [118,119]. Inositol phosphate metabolism has been linked with telomere homeostasis in other eukaryotes [120], but may have specific features in *T. brucei*, given the potentially novel role of PolIE in telomere protection and VSG switching [57••,59••], as well as the recent demonstration that at least one telomere-binding factor also binds telomeric RNA [121•].

A variant-surface glycoprotein-expression factory

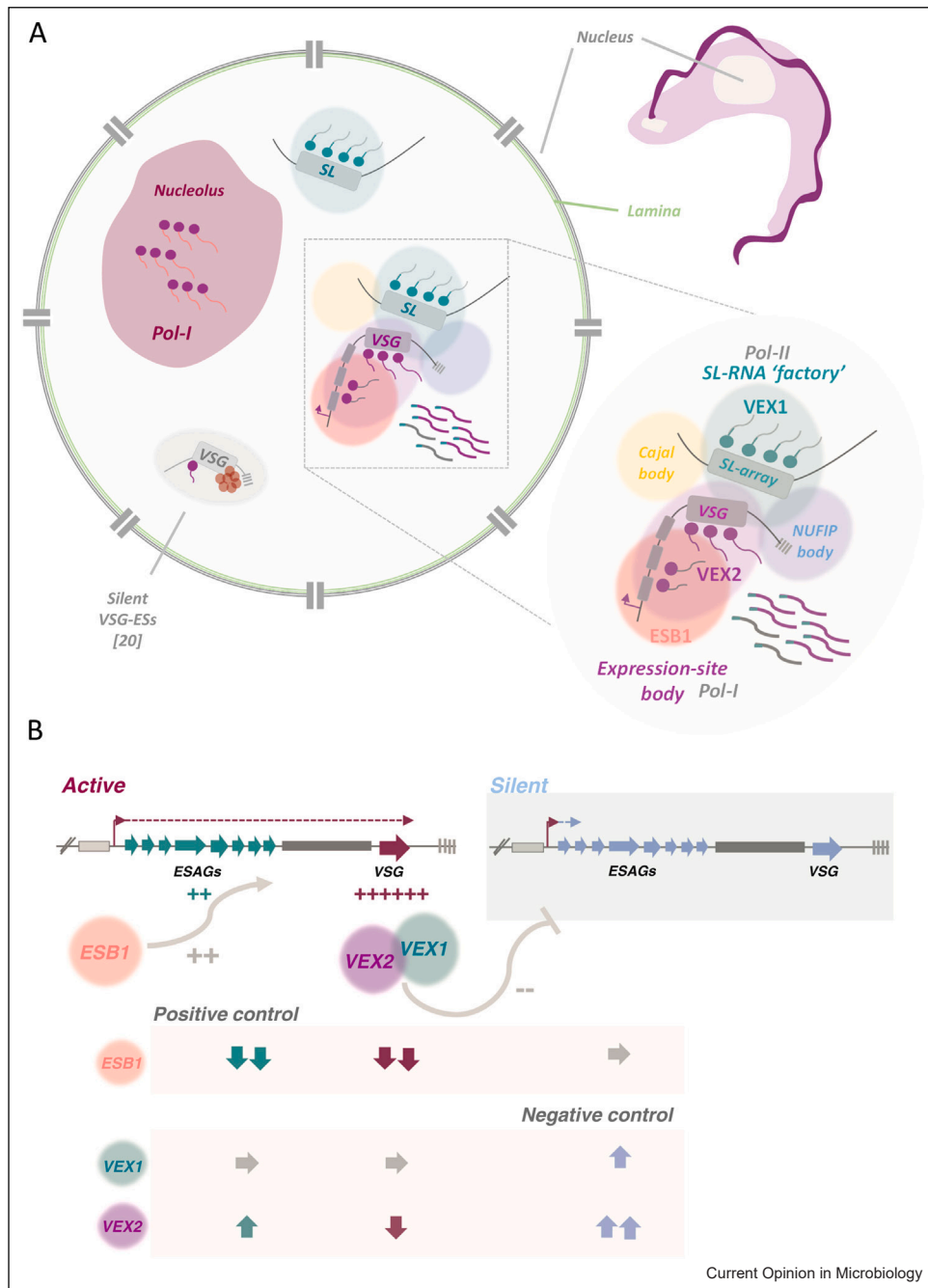
Nearly 20 years after the ESB was observed, the first specifically associated factors were discovered. VSG-exclusion protein 1 (VEX1) was identified through a genome-wide RNAi screen and shown to form 1 or 2 discrete subnuclear protein condensates, one

immediately adjacent to the ESB (Figure 4) [122]. VEX1 depletion leads to derepression of silent VSG ESs in ~25% of cells [122]. Affinity purification of VEX1 later identified VEX2 (Figure 4), an exceptionally large putative RNA helicase that forms a single nuclear condensate colocalising with the ESB [123••]. VEX2 depletion causes the ESB to disperse and all VSG ESs are simultaneously transcribed (including metacyclic VSGs), with >95% of the cells displaying multiple VSG coats. Depletion of neither VEX1 nor VEX2 results in detectable change in Pol-II transcripts, but increased Pol-I-transcribed procyclin was observed after VEX2 loss, indicating some effects beyond the VSG ESs [122,123••]. The VEX complex also interacts with the conserved chromatin-assembly factor, CAF-1 [123••], whose depletion leads to derepression of silent VSGs during S-phase. CAF-1 is a histone chaperone responsible for loading H3–H4 dimers onto newly synthesised DNA strands during DNA replication, and transient VEX/CAF-1 interaction is required for VEX reassembly and VSG-exclusion inheritance.

T. brucei mRNA, such as nearly all kinetoplastid protein-coding genes, is matured by *trans*-splicing of the 5' splice leader (SL)-RNA cap, which is transcribed from array sequences. Interestingly, the active VSG forms a stable interchromosomal interaction with one SL-array to sustain VSG monogenic expression and efficient mRNA splicing (Figure 4) [105••]. In fact, VEX1 and VEX2 primarily associate with the active VSG transcription compartment and the SL-RNA compartment, respectively, therefore acting as a supramolecular interchromosomal bridge (Figure 4). Strikingly, VEX2 sustains this interaction with just the active VSG ES, as in its absence all VSG ESs can access the SL-array. Additionally, VEX2 knockdown causes upregulation of expression-site-associated genes (ESAGs) located upstream of the ES VSG and normally expressed at lower levels than the active VSG [105••]. Given VEX2 depletion also increases association between the ES promoter or ESAGs with the SL-array, it is tempting to speculate that VEX2 maximises the interaction between the VSG gene itself and the SL-array, to fine-tune VSG expression [105••].

Further activities are emerging that may relate to the ESB. A highly SUMOylated subnuclear focus has been shown to colocalise with the active ES. More recently, the first VSG ES transcriptional activator, ESB1, was identified through a protein-localisation screen of bloodstream-form cells [124••]. ESB1 forms a discrete protein condensate that colocalises with the ESB, and when depleted leads to a transcriptional drop at the active VSG ES, whilst 'silent' VSG ESs remain unaffected. Conversely, its overexpression activates inactive VSG promoters. If, and how, this activity interdigitates with VEX is currently unknown. Two additional nuclear bodies, a NUFIP body and a Cajal body, likely involved

Figure 4



Spatial integration of transcription and splicing sustains VSG monoallelic expression in *T. brucei* bloodstream forms. **(a)** The single active VSG is transcribed within the ESB and establishes a stable interchromosomal interaction with one of the SL-arrays. VEX2 and VEX1 form discrete protein condensates that associate with the active VSG and the SL-arrays, respectively. VEX2 sustains the exclusive interaction between a single VSG ES and the SL-arrays, and following its depletion, all VSG ESs can access the SL-arrays and are derepressed. ESB1 is a stage-specific transcriptional activator whose depletion leads to a transcriptional drop at the active ES. Moreover, the active VSG ES resides within a highly SUMOylated focus [138] and TDP1, a high-mobility-group box protein, is enriched at this site and facilitates Pol-I transcription [139]. Only VEX2 and ESB1 are highlighted in the figure because those are the only two factors known to specifically associate with the ESB to date. Two additional nuclear bodies likely to be involved in RNA processing/splicing associate with the ESB and the SL-factory (Cajal and NUFIP bodies). Furthermore, the silent ESs display more peripheral nuclear locations: several repressing factors (red circles) associated with heterochromatin formation (including chromatin remodellers, histone chaperones, histone modifiers and components of the nuclear lamina) and telomere stability sustain their 'inactive' state. **(b)** ESB1 and VEX1/VEX2 are positive and negative regulators of VSG expression, respectively. ESB1 depletion leads to a transcriptional drop at the active ES without any effect on distal VSG loci, whereas VEX depletion, especially VEX2, leads to derepression of silent VSGs. Moreover, VEX2 depletion leads to an increase in the expression of ESAGs from the active ES, though usually much less abundantly than the active VSG.

in splicing/RNA processing, have been shown to associate with the ESB and *SL*-array [106••]. Thus, trypanosomes assemble a conglomerate of nuclear bodies — a ‘production line’ — to boost the expression of their active *VSG*.

Function, conservation and specialisation

Interchromosomal interactions are usually stochastic, with very few stably persisting, though prolonged interactions between superenhancers are known to boost transcription of monogenically expressed immunoglobulin heavy chains and olfactory receptors [125,126]. The association between the active *VSG* and the *SL*-array appears reminiscent of such enhancer interactions but emerges post-transcriptionally and is currently the only example amongst unicellular organisms. Intriguingly, CAF-1 has been implicated in epigenetic control of olfactory-receptor monogenic expression [127].

ESB1 and VEX play vital yet distinct roles in *VSG*-expression control, consistent with positive and negative regulation, respectively. Given that VEX disperses rapidly following Pol-I transcription and splicing inhibition [105••,123••], one could speculate that transcription of ES-derived mRNA, possibly through activation by ESB1, precedes VEX association with *VSG* loci. Interestingly, forced expression of two *VSG*s simultaneously results in dynamic colocalisation with the ESB [128,129]. Furthermore, *VSG* ESs may stochastically interact with the *SL*-arrays, competing for a limited pool of VEX2, and thus stabilising an exclusive interaction between a single *VSG* locus and the *SL*-array. In this regard, a family of RNA helicases has been identified as regulators of RNA-containing membraneless organelles, controlling RNA flux into and out of these bodies and therefore enabling spatial and temporal control of various RNA-processing steps [130••]. Perhaps paradoxically, however, expression of a second *VSG* from a ribosomal locus results in an enlarged VEX2 focus [131]. Notably, it was not clear whether VEX2 was upregulated, which would argue that it is not limiting in the cell, or simply less condensed and spread across a wider region. Uncovering the interplay between the various ES-associated nuclear bodies and identifying factors beyond ESB1 and VEX1/2, is needed to understand how specific the *T. brucei* *VSG* monogenic machinery is, and may answer questions regarding the assembly, inheritance and developmental regulation of this complex nuclear organisation.

Remarkably, ESB1, VEX1 and VEX2 homologues are found across kinetoplastids. VEX1 contains a conserved SWIM-type Zn finger at the C-terminus, though the remaining sequence is most highly conserved amongst trypanosomes that undergo antigenic variation [122]. VEX2 contains N-terminal alpha-solenoid motifs, usually present in ring-forming proteins (including

several components of the nucleopore complex), which are often involved in protein–nucleic acid interactions, and a putative RNA helicase at the C-terminus [123••]. The helicase core is related to UPF1, a widely conserved RNA helicase involved in nonsense-mediated decay as well as cotranscriptional RNA surveillance [132,133]. Why VEX2 resembles UPF1 is intriguing as classical nonsense-mediated decay appears absent in trypanosomes [134], though UPF1 has been implicated in Xist regulation and chromosome-X inactivation in mammals [135]. African and American trypanosomes encode both a canonical UPF1 and a UPF1-related helicase domain in VEX2, whereas *Leishmania*, which does not undergo antigenic variation, only possesses the UPF1-related helicase [123••]. Understanding what these proteins do in *Leishmania* could shed light on the co-opting of this factor for antigenic variation control in *T. brucei*, while the presence of a likely VEX2 orthologue in *T. cruzi* may suggest a need for multigene-expression control.

Conclusions and perspectives

Understanding of the mechanisms and underlying machinery directing *VSG*-expression control and switching during antigenic variation continues to grow (Figures 3b and 4), but remains incomplete. Notably, whilst sub-nuclear processes and structures directing selection and maintenance of a single active *VSG* allele are emerging, how and when monogenic control is passed between two *VSG* ESs to execute a transcriptional switch awaits clarification. One possibility is that DNA breaks or replication might liberate ESB1 and VEX2, allowing other *VSG*s to compete for these factors and for *SL*-RNA interaction. Alternatively, mechanical stress caused by trypanosome migration within the host and tissues could disrupt nuclear architecture and result in similar disassociation. In terms of *VSG* recombination, though considerable evidence points to harnessing HR to execute activation of a functional silent *VSG* gene, definitive demonstration of the pathway directing mosaic *VSG* formation, including abundant *VSG* pseudogenes, is lacking. Moreover, the three competing models for *VSG*-recombination initiation need further tests. For instance, what is known about the direction, rate and nature of early replication of the active *VSG* ES: is this directed by the origin-recognition complex that acts throughout the genome [136,137], or might it be explained by DNA PolIE [57••,59••]? More broadly, mechanisms of antigenic variation have not yet been explored in extra-vascular trypanosomes in the host, where a greater diversity of expressed *VSG*s is seen (BioRxiv 10.1101/2022.06.27.497797v1). Investigations to date have not revealed truly trypanosome-specific pathways or factors that have emerged to serve antigenic variation, but instead the co-option and repurposing of more widely conserved activities, whether across trypanosomatids, kinetoplastids or even more widely. Thus, the likelihood

of developing a specific ‘anti-VSG switching’ compound or drug currently appears slim. Nonetheless, evidence from the study of cancer may offer hope, since dividing and metastasizing cancer cells have been shown to be vulnerable when targeting conserved activities that are also active in non-cancer cells and tissues.

CRedit authorship contribution statement

Joana Faria: Conceptualisation, Writing – original draft, Writing – review & editing, Funding acquisition. **Emma Briggs:** Conceptualisation, Writing – original draft, Writing – review & editing, Funding acquisition. **Jennifer Black:** Conceptualisation, Writing – original draft, Writing – review & editing. **Richard McCulloch:** Conceptualisation, Writing – original draft, Writing – review & editing, Funding acquisition.

Conflict of interest statement

All authors declare they have no conflict of interest.

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