
Article:
Correia Faria, Joana Raquel orcid.org/0000-0001-6274-8143, Briggs, Emma, Black, Jennifer Ann et al. (1 more author) (2022) Emergence and adaptation of the cellular machinery directing antigenic variation in the African trypanosome. Current Opinion in Microbiology. 102209. ISSN 1369-5274

https://doi.org/10.1016/j.mib.2022.102209

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

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

Joana Faria[1], Emma M Briggs[2,4], Jennifer A Black[3,4] and Richard McCulloch[4]

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' *VSG*s 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.

Addresses
[1] York Biomedical Research Institute, Department of Biology, University of York, United Kingdom
[2] Institute for Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom
[3] Department of Cell and Molecular Biology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil
[4] Wellcome Centre for Integrative Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Sir Graeme Davies Building, 120 University Place, Glasgow G12 8TA, United Kingdom

Corresponding authors: Joana Faria (joana.correiafaria@york.ac.uk), Richard McCulloch (richard.mcculloch@glasgow.ac.uk)

Current Opinion in Microbiology 2022, **70**:102209

This review comes from a themed issue on **Host-Microbe Interactions: Parasites**

Edited by **Christian Doerig** and **Debopam Chakrabarti**

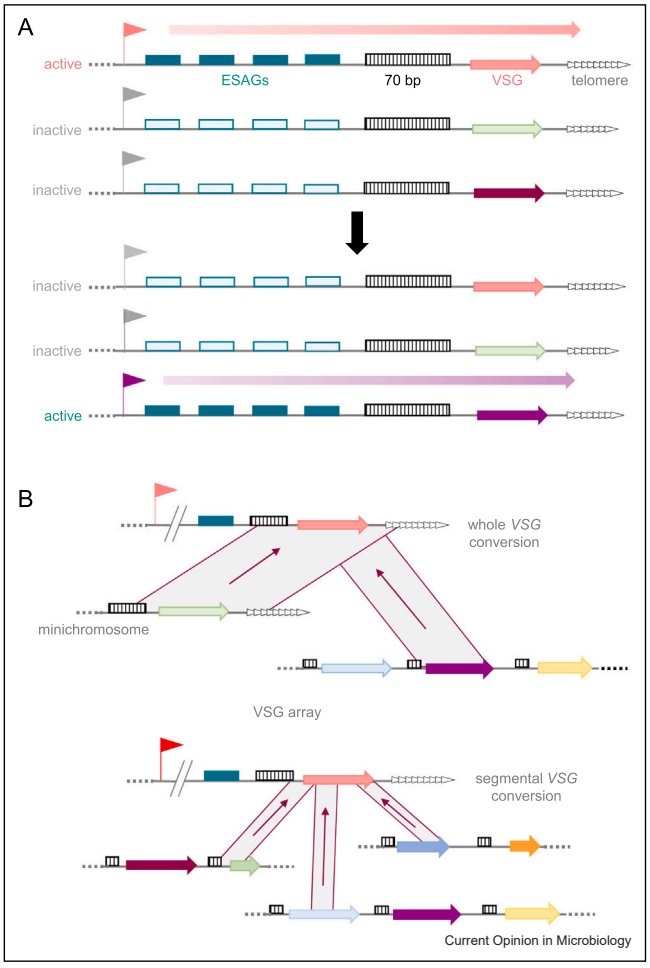
https://doi.org/10.1016/j.mib.2022.102209

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 *VSG*s 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) *VSG*s, 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*.

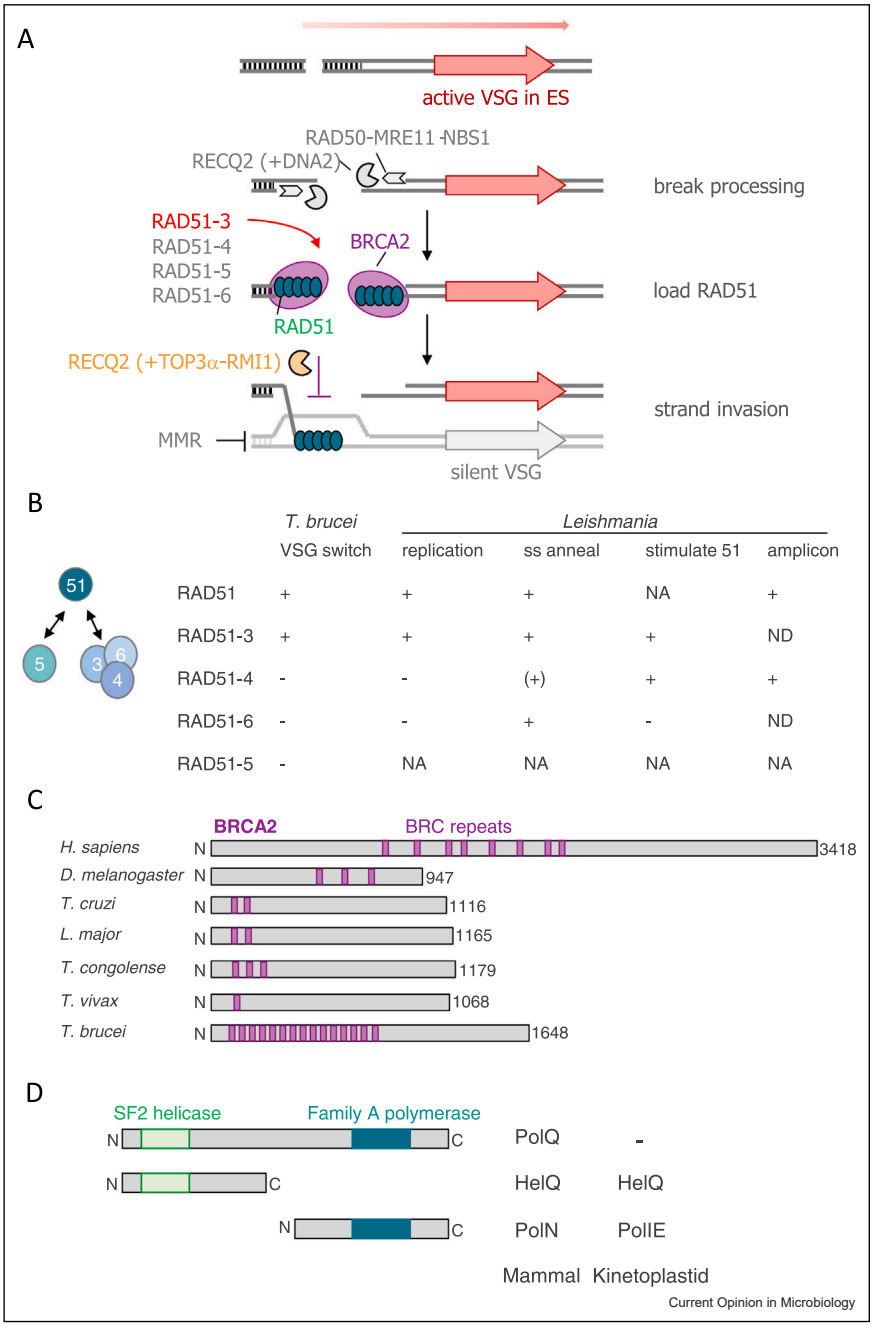
Though there appears to be little need for new therapies against human African trypanosomiasis, given recent dramatic successes in controlling case numbers [2], impeding antigenic variation would theoretically provide a highly effective strategy to tackle disease causing any pathogen that uses this form 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 homoeostasis [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' *VSG*s, which are distinct from any single (pseudo)gene 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 *VSG*s 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.

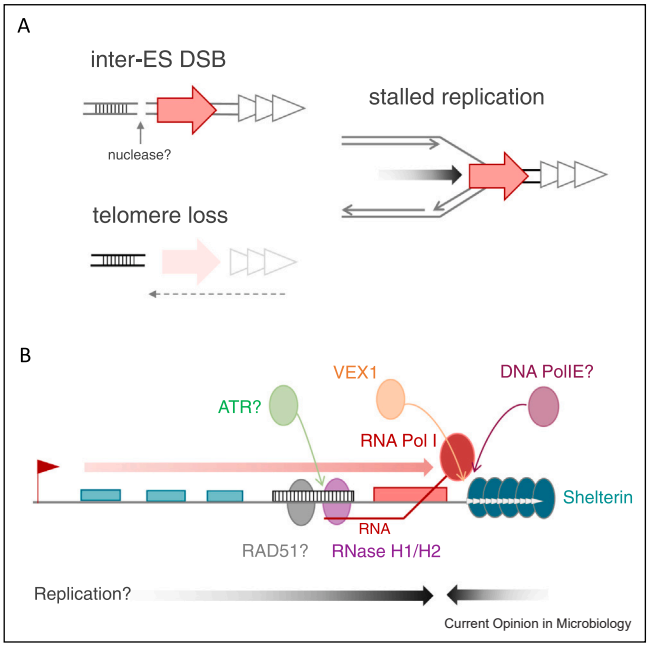
In all kinetoplastids tested so far [42–47], no evidence has emerged for the use of Ku-directed NHEJ [48•]. Instead, untemplated DNA DSB repair relies on MMEJ or single-strand annealing (SSA, relying on longer stretches of homology than the imperfect ~5–20-bp homology used in MMEJ). MMEJ in metazoans 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 PolI-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 homoeostasis [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 *VSG*s (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 homoeostasis 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•].

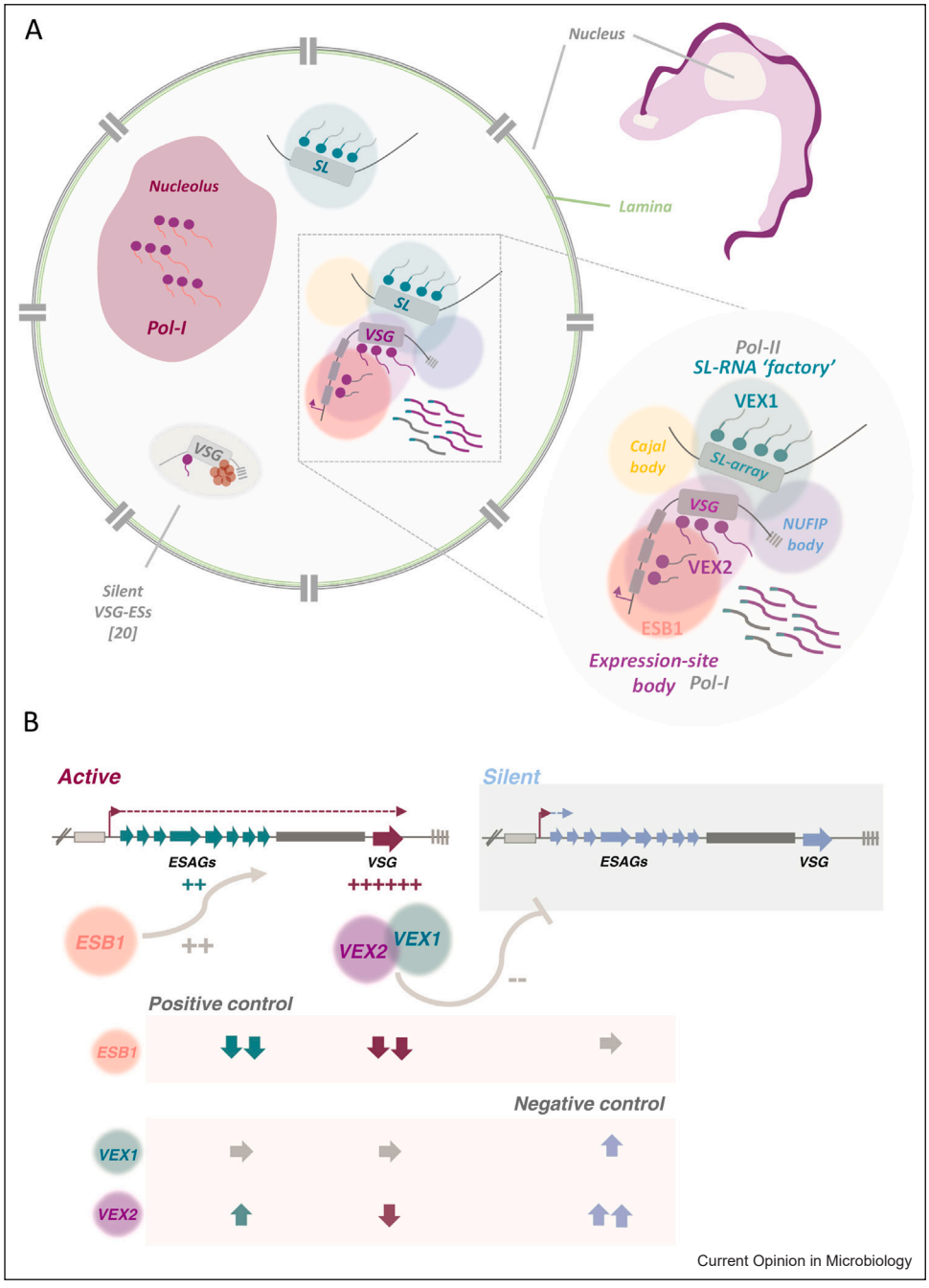
A variant-surface glycoprotein-expression factory

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 promotor 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*-array, respectively. VEX2 sustains the exclusive interaction between a single *VSG* ES and the *SL*-array, 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 immunoglobin 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 subnuclear 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 dissociation. 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 extravascular trypanosomes in the host, where a greater diversity of expressed VSGs 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.

Acknowledgements

Work in RMcC's lab is supported by a Wellcome Trust Investigator Award [224501/Z/21/Z] and by the Biotechnology and Biological Sciences Research Council (BBSRC) [BB/N016165/1, BB/R017166/1, BB/W001101/1]. EB is the recipient of a Wellcome Trust Sir Henry Wellcome Fellowship [218648/Z/19/Z], and JF is the recipient of a Wellcome Trust Sir Henry Dale Fellowship [222573/Z/21/Z]. JB is the recipient of a FAPESP Post-Doctoral Fellowship [2020/01883-7]. The Wellcome Centre for Integrative Parasitology is supported by core funding from the Wellcome Trust [104111].

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest.

1. Deitsch KW, Lukehart SA, Stringer JR: **Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens**. *Nat Rev Microbiol* 2009, **7**:493-503.
2. Franco JR, Cecchi G, Paone M, Diarra A, Grout L, Kadima Ebeja A, Simarro PP, Zhao W, Argaw D: **The elimination of human African trypanosomiasis: achievements in relation to WHO road map targets for 2020**. *PLoS Negl Trop Dis* 2022, **16**:e0010047.
3. Sheader K, Vaughan S, Minchin J, Hughes K, Gull K, Rudenko G: **Variant surface glycoprotein RNA interference triggers a precytokinesis cell cycle arrest in African trypanosomes**. *Proc Natl Acad Sci USA* 2005, **102**:8716-8721.
4. Aresta-Branco F, Sanches-Vaz M, Bento F, Rodrigues JA, Figueiredo LM: **African trypanosomes expressing multiple VSGs are rapidly eliminated by the host immune system**. *Proc Natl Acad Sci USA* 2019, **116**:20725-20735.
5. Borst P: **Antigenic variation in eukaryotic parasites**. In *Mobile DNA II*. Edited by Craig NL, Berg DE. ASM Press; 2002:953-971.
6. Borst P, Cross GA: **Molecular basis for trypanosome antigenic variation**. *Cell* 1982, **29**:291-303.
7. McCulloch R, Morrison LJ, Hall JP: **DNA recombination strategies during antigenic variation in the African trypanosome**. *Microbiol Spectr* 2015, **3**:MDNA3-0016-2014.
8. Bonilla B, Hengel SR, Grundy MK, Bernstein KA: **RAD51 gene family structure and function**. *Annu Rev Genet* 2020, **54**:25-46.
9. McCulloch R, Barry JD: **A role for RAD51 and homologous recombination in *Trypanosoma brucei* antigenic variation**. *Genes Dev* 1999, **13**:2875-2888.
10. Hartley CL, McCulloch R: ***Trypanosoma brucei* BRCA2 acts in antigenic variation and has undergone a recent expansion in BRC repeat number that is important during homologous recombination**. *Mol Microbiol* 2008, **68**:1237-1251.
11. Trenaman A, Hartley C, Prorocic M, Passos-Silva DG, van den Hoek M, Nechyporuk-Zloy V, Machado CR, McCulloch R: ***Trypanosoma brucei* BRCA2 acts in a life cycle-specific genome stability process and dictates BRC repeat number-dependent RAD51 subnuclear dynamics**. *Nucleic Acids Res* 2013, **41**:943-960.
12. Proudfoot C, McCulloch R: **Distinct roles for two RAD51-related genes in *Trypanosoma brucei* antigenic variation**. *Nucleic Acids Res* 2005, **33**:6906-6919.
13. Dobson R, Stockdale C, Lapsley C, Wilkes J, McCulloch R: **Interactions among *Trypanosoma brucei* RAD51 paralogues in DNA repair and antigenic variation**. *Mol Microbiol* 2011, **81**:434-456.
14. Conway C, McCulloch R, Ginger ML, Robinson NP, Browitt A, Barry JD: **Ku is important for telomere maintenance, but not for differential expression of telomeric VSG genes, in African trypanosomes**. *J Biol Chem* 2002, **277**:21269-21277.
15. Janzen CJ, Lander F, Dreesen O, Cross GA: **Telomere length regulation and transcriptional silencing in KU80-deficient *Trypanosoma brucei***. *Nucleic Acids Res* 2004, **32**:6575-6584.
16. Devlin R, Marques CA, McCulloch R: **Does DNA replication direct locus-specific recombination during host immune evasion by antigenic variation in the African trypanosome?** *Curr Genet* 2017, **63**:441-449.
17. Obergfell KP, Seifert HS: **Mobile DNA in the pathogenic Neisseria**. *Microbiol Spectr* 2015, **3**:MDNA3-0015-2014.
18. Demeyer A, Benhelli-Mokrani H, Chenais B, Weigel P, Fleury F: **Inhibiting homologous recombination by targeting RAD51 protein**. *Biochim Biophys Acta Rev Cancer* 2021, **1876**:188597.
19. Orhan E, Velazquez C, Tabet I, Sardet C, Theillet C: **Regulation of RAD51 at the transcriptional and functional levels: what prospects for cancer therapy?** *Cancers* 2021, **13**:2930.
20. Zhang WW, Lypaczewski P, Matlashewski G: **Optimized CRISPR-Cas9 genome editing for *Leishmania* and its use to target a multigene family, induce chromosomal translocation, and study DNA break repair mechanisms**. *mSphere* 2017, **2**:e00340-16.
21. Kelso AA, Waldvogel SM, Luthman AJ, Sehorn MG: **Homologous recombination in protozoan parasites and recombinase inhibitors**. *Front Microbiol* 2017, **8**:1716.
22. Toh M, Ngeow J: **Homologous recombination deficiency: cancer predispositions and treatment implications**. *Oncologist* 2021, **26**:e1526-e1537.
23. Hall M 3rd, Misra S, Chaudhuri M, Chaudhuri G: **Peptide aptamer mimicking RAD51-binding domain of BRCA2 inhibits DNA damage repair and survival in Trypanosoma brucei**. *Microb Pathog* 2011, **50**:252-262.
24. • Pantelejevs T, Hyvonen M: **Divergent binding mode for a protozoan BRC repeat to RAD51**. *Biochem J* 2022, **479**:1031-1043.
The most recent paper dissecting novelty in kinetoplastid BRCA2-RAD51 interaction, following from distinct observations in previous work by Hartley et al., 2008, Hall et al., 2011 and Trenaman et al., 2013.
25. Genois MM, Mukherjee A, Ubeda JM, Buisson R, Paquet E, Roy G, Plourde M, Coulombe Y, Ouellette M, Masson JY: **Interactions between BRCA2 and RAD51 for promoting homologous recombination in *Leishmania* infantum**. *Nucleic Acids Res* 2012, **40**:6570-6584.
26. Scott DE, Francis-Newton NJ, Marsh ME, Coyne AG, Fischer G, Moschetti T, Bayly AR, Sharpe TD, Haas KT, Barber L, *et al.*: **A small-molecule inhibitor of the BRCA2-RAD51 interaction modulates RAD51 assembly and potentiates DNA damage-induced cell death**. *Cell Chem Biol* 2021, **28**:835-847.e835.
27. Rein HL, Bernstein KA, Baldock RA: **RAD51 paralog function in replicative DNA damage and tolerance**. *Curr Opin Genet Dev* 2021, **71**:86-91.
28. Berti M, Teloni F, Mijic S, Ursich S, Fuchs J, Palumbieri MD, Krietsch J, Schmid JA, Garcin EB, Gon S, *et al.*: **Sequential role of RAD51**

paralog complexes in replication fork remodeling and restart. *Nat Commun* 2020, **11**:3531.

29. Rosenbaum JC, Bonilla B, Hengel SR, Mertz TM, Herken BW, Kazemier HG, Pressimone CA, Ratterman TC, MacNary E, De Magis A, *et al.*: **The Rad51 paralogs facilitate a novel DNA strand specific damage tolerance pathway**. *Nat Commun* 2019, **10**:3515.

30. Mohan M, Akula D, Dhillon A, Goyal A, Anindya R: **Human RAD51 paralogue RAD51C fosters repair of alkylated DNA by interacting with the ALKBH3 demethylase**. *Nucleic Acids Res* 2019, **47**:11729-11745.

31. Genois MM, Plourde M, Ethier C, Roy G, Poirier GG, Ouellette M, Masson JY: **Roles of Rad51 paralogs for promoting homologous recombination in *Leishmania* infantum**. *Nucleic Acids Res* 2015, **43**:2701-2715.

32. •• Damasceno JD, Reis-Cunha J, Crouch K, Beraldi D, Lapsley C, Tosi LRO, Bartholomeu D, McCulloch R: **Conditional knockout of RAD51-related genes in *Leishmania* major reveals a critical role for homologous recombination during genome replication**. *PLoS Genet* 2020, **16**:e1008828.

This study reveals differential roles for RAD51 and the RAD51 paralogues in *Leishmania* DNA repair and replication, and demonstrates that DNA replication in *Leishmania* requires HR activity, which has relevance for T. brucei DSB repair and antigenic variation.

33. Barbet AF, Kamper SM: **The importance of mosaic genes to trypanosome survival**. *Parasitol Today* 1993, **9**:63-66.

34. Kamper SM, Barbet AF: **Surface epitope variation via mosaic gene formation is potential key to long-term survival of *Trypanosoma brucei***. *Mol Biochem Parasitol* 1992, **53**:33-44.

35. Roth C, Bringaud F, Layden RE, Baltz T, Eisen H: **Active late-appearing variable surface antigen genes in *Trypanosoma equiperdum* are constructed entirely from pseudogenes**. *Proc Natl Acad Sci USA* 1989, **86**:9375-9379.

36. Hall JP, Wang H, Barry JD: **Mosaic VSGs and the scale of *Trypanosoma brucei* antigenic variation**. *PLoS Pathog* 2013, **9**:e1003502.

37. Mugnier MR, Cross GA, Papavasiliou FN: **The in vivo dynamics of antigenic variation in *Trypanosoma brucei***. *Science* 2015, **347**:1470-1473.

38. •• Jayaraman S, Harris C, Paxton E, Donachie AM, Vaikkinen H, McCulloch R, Hall JPJ, Kenny J, Lenzi L, Hertz-Fowler C, *et al.*: **Application of long read sequencing to determine expressed antigen diversity in *Trypanosoma brucei* infections**. *PLoS Negl Trop Dis* 2019, **13**:e0007262.

The most recent demonstration of expressed VSG diversity during infections, following from earlier work by Hall et al, 2013 and Mugnier et al., 2015.

39. McCulloch R, Field MC: **Quantitative sequencing confirms VSG diversity as central to immune evasion by *Trypanosoma brucei***. *Trends Parasitol* 2015, **31**:346-349.

40. Marcello L, Barry JD: **Analysis of the VSG gene silent archive in *Trypanosoma brucei* reveals that mosaic gene expression is prominent in antigenic variation and is favored by archive substructure**. *Genome Res* 2007, **17**:1344-1352.

41. Glover L, Jun J, Horn D: **Microhomology-mediated deletion and gene conversion in African trypanosomes**. *Nucleic Acids Res* 2011, **39**:1372-1380.

42. Conway C, Proudfoot C, Burton P, Barry JD, McCulloch R: **Two pathways of homologous recombination in *Trypanosoma brucei***. *Mol Microbiol* 2002, **45**:1687-1700.

43. Burton P, McBride DJ, Wilkes JM, Barry JD, McCulloch R: **Ku heterodimer-independent end joining in *Trypanosoma brucei* cell extracts relies upon sequence microhomology**. *Eukaryot Cell* 2007, **6**:1773-1781.

44. Glover L, McCulloch R, Horn D: **Sequence homology and microhomology dominate chromosomal double-strand break repair in African trypanosomes**. *Nucleic Acids Res* 2008, **36**:2608-2618.

45. Zhang WW, Matlashewski G: **CRISPR-Cas9-mediated genome editing in *Leishmania donovani***. *MBio* 2015, **6**:e00861.

46. •• Zhang WW, Matlashewski G: **Single-strand annealing plays a major role in double-strand DNA break repair following CRISPR-Cas9 cleavage in *Leishmania***. *mSphere* 2019, **4**:e00408-19.

First demonstration of a role for kinetoplastid HelQ in DNA DSB repair.

47. Peng D, Kurup SP, Yao PY, Minning TA, Tarleton RL: **CRISPR-Cas9-mediated single-gene and gene family disruption in *Trypanosoma cruzi***. *MBio* 2015, **6**:e02097-02014.

48. • Nenarokova A, Zahonova K, Krasilnikova M, Gahura O, McCulloch R, Zikova A, Yurchenko V, Lukes J: **Causes and effects of loss of classical nonhomologous end joining pathway in parasitic eukaryotes**. *mBio* 2019, **10**:e01541-9.

A comparison of the presence and absence of the Ku heterodimer across kinetoplastids and analysis of genome differences where Ku is absent or present.

49. Brambati A, Barry RM, Sfeir A: **DNA polymerase theta (Poltheta) - an error-prone polymerase necessary for genome stability**. *Curr Opin Genet Dev* 2020, **60**:119-126.

50. Kent T, Chandramouly G, McDevitt SM, Ozdemir AY, Pomerantz RT: **Mechanism of microhomology-mediated end-joining promoted by human DNA polymerase theta**. *Nat Struct Mol Biol* 2015, **22**:230-237.

51. Kent T, Mateos-Gomez PA, Sfeir A, Pomerantz RT: **Polymerase theta is a robust terminal transferase that oscillates between three different mechanisms during end-joining**. *Elife* 2016, **5**:e13740.

52. • Wang Z, Song Y, Li S, Kurian S, Xiang R, Chiba T, Wu X: **DNA polymerase theta (POLQ) is important for repair of DNA double-strand breaks caused by fork collapse**. *J Biol Chem* 2019, **294**:3909-3919.

Provides an important demonstration of the range of activities provided by PolQ in some eukaryotes.

53. Ceccaldi R, Liu JC, Amunugama R, Hajdu I, Primack B, Petalcorin MI, O'Connor KW, Konstantinopoulos PA, Elledge SJ, Boulton SJ, *et al.*: **Homologous-recombination-deficient tumours are dependent on Poltheta-mediated repair**. *Nature* 2015, **518**:258-262.

54. Wood RD, Doublie S: **DNA polymerase theta (POLQ), double-strand break repair, and cancer**. *DNA Repair* 2016, **44**:22-32.

55. McVey M, Lee SE: **MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings**. *Trends Genet* 2008, **24**:529-538.

56. Yousefzadeh MJ, Wood RD: **DNA polymerase POLQ and cellular defense against DNA damage**. *DNA Repair* 2013, **12**:1-9.

57. •• Leal AZ, Schwebs M, Briggs E, Weisert N, Reis H, Lemgruber L, Luko K, Wilkes J, Butter F, McCulloch R, *et al.*: **Genome maintenance functions of a putative *Trypanosoma brucei* translesion DNA polymerase include telomere association and a role in antigenic variation**. *Nucleic Acids Res* 2020, **48**:9660-9680.

Analysis of the unusual evolutionary history of a putative PolQ-like DNA polymerase in *T. brucei*, and demonstration of a role in VSG switching and gene expression.

58. Fernandez-Orgiler A, Martinez-Jimenez MI, Alonso A, Alcolea PJ, Requena JM, Thomas MC, Blanco L, Larraga V: **A putative *Leishmania* DNA polymerase theta protects the parasite against oxidative damage**. *Nucleic Acids Res* 2016, **44**:4855-4870.

59. •• Rabbani MAG, Tonini ML, Afrin M, Li B: **POLIE suppresses telomerase-mediated telomere G-strand extension and helps ensure proper telomere C-strand synthesis in trypanosomes**. *Nucleic Acids Res* 2022, **50**:2036-2050.

Demonstration of a role for a putative PolQ-like DNA polymerase in the mainatenance of telomeres in *T. brucei*.

60. de Lima LP, Calderano SG, da Silva MS, de Araujo CB, Vasconcelos EJR, Iwai LK, Pereira CA, Fragoso SP, Elias MC: **Ortholog of the polymerase theta helicase domain modulates DNA replication in *Trypanosoma cruzi***. *Sci Rep* 2019, **9**:2888.

61. Kamp JA, Lemmens B, Romeijn RJ, Changoer SC, van Schendel R, Tijsterman M: **Helicase Q promotes homology-driven DNA double-strand break repair and prevents tandem duplications**. *Nat Commun* 2021, **12**:7126.

62. Zhou J, Gelot C, Pantelidou C, Li A, Yucel H, Davis RE, Farkkila A, Kochupurakkal B, Syed A, Shapiro GI, *et al.*: **A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors**. *Nat Cancer* 2021, **2**:598-610.

63. Meyer D, Fu BX, Heyer WD: **DNA polymerases delta and lambda cooperate in repairing double-strand breaks by microhomology-mediated end-joining in *Saccharomyces cerevisiae***. *Proc Natl Acad Sci USA* 2015, **112**:E6907-6916.

64. Turner CM, Barry JD: **High frequency of antigenic variation in *Trypanosoma brucei* rhodesiense infections**. *Parasitology* 1989, **99**:67-75.

65. da Silva MS, Hovel-Miner GA, Briggs EM, Elias MC, McCulloch R: **Evaluation of mechanisms that may generate DNA lesions triggering antigenic variation in African trypanosomes**. *PLoS Pathog* 2018, **14**:e1007321.

66. Barry JD: **The relative significance of mechanisms of antigenic variation in African trypanosomes**. *Parasitol Today* 1997, **13**:212-218.

67. Borst P, Rudenko G, Taylor MC, Blundell PA, van Leeuwen F, Bitter W, Cross M, McCulloch R: **Antigenic variation in trypanosomes**. *Arch Med Res* 1996, **27**:379-388.

68. Barry JD, McCulloch R: **Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite**. *Adv Parasitol* 2001, **49**:1-70.

69. Boothroyd CE, Dreesen O, Leonova T, Ly KI, Figueiredo LM, Cross GA, Papavasiliou FN: **A yeast-endonuclease-generated DNA break induces antigenic switching in *Trypanosoma brucei***. *Nature* 2009, **459**:278-281.

70. Hovel-Miner G, Mugnier MR, Goldwater B, Cross GA, Papavasiliou FN: **A conserved DNA repeat promotes selection of a diverse repertoire of *Trypanosoma brucei* surface antigens from the genomic archive**. *PLoS Genet* 2016, **12**:e1005994.

71. Glover L, Alsford S, Horn D: **DNA break site at fragile subtelomeres determines probability and mechanism of antigenic variation in African trypanosomes**. *PLoS Pathog* 2013, **9**:e1003260.

72. •• Thivolle A, Mehnert AK, Tihon E, McLaughlin E, Dujeancourt-Henry A, Glover L: **DNA double strand break position leads to distinct gene expression changes and regulates VSG switching pathway choice**. *PLoS Pathog* 2021, **17**:e1010038.

The authors investigate different locations of DSBs within the active *VSG* ES and how they influence VSG switching, showing that the location of the DSB influences the choice of repair pathway used and that error prone repair dominates DSB resolution in the active ES. An important refinement in understanding how DNA DSBs act as triggers for VSG switching in African trypanosomes.

73. Devlin R, Marques CA, Paape D, Prorocic M, Zurita-Leal AC, Campbell SJ, Lapsley C, Dickens N, McCulloch R: **Mapping replication dynamics in Trypanosoma brucei reveals a link with telomere transcription and antigenic variation**. *Elife* 2016, **5**:e12765.

74. • Glover L, Marques CA, Suska O, Horn D: **Persistent DNA damage *Foci* and DNA replication with a broken chromosome in the African trypanosome**. *MBio* 2019, **10**:e01252-19.

Demonstration of unusual tolerance of *T. brucei* to unrepaired DNA breaks during cell cycle progression

75. Robinson NP, McCulloch R, Conway C, Browitt A, Barry JD: **Inactivation of Mre11 does not affect VSG gene duplication mediated by homologous recombination in *Trypanosoma brucei***. *J Biol Chem* 2002, **277**:26185-26193.

76. •• Mehnert AK, Prorocic M, Dujeancourt-Henry A, Hutchinson S, McCulloch R, Glover L: **The MRN complex promotes DNA repair by homologous recombination and restrains antigenic variation in African trypanosomes**. *Nucleic Acids Res* 2021, **49**:1436-1454.

Refined understanding of how DNA DSBs are processed in the VSG ES in *T. brucei*, and the consequences of MRN inactivation for VSG switching.

77. Kim HS, Cross GA: **TOPO3alpha influences antigenic variation by monitoring expression-site-associated VSG switching in *Trypanosoma brucei***. *PLoS Pathog* 2010, **6**:e1000992.

78. Kim HS, Cross GA: **Identification of *Trypanosoma brucei* RMI1/BLAP75 homologue and its roles in antigenic variation**. *PLoS One* 2011, **6**:e25313.

79. Cohen S, Guenole A, Lazar I, Marnef A, Clouaire T, Vernekar DV, Puget N, Rocher V, Arnould C, Aguirrebengoa M, *et al.*: **A POLD3/BLM dependent pathway handles DSBs in transcribed chromatin upon excessive RNA:DNA hybrid accumulation**. *Nat Commun* 2022, **13**:2012.

80. Harami GM, Palinkas J, Seol Y, Kovacs ZJ, Gyimesi M, Harami-Papp H, Neuman KC, Kovacs M: **The toposiomerase IIIalpha-RMI1-RMI2 complex orients human Bloom's syndrome helicase for efficient disruption of D-loops**. *Nat Commun* 2022, **13**:654.

81. Xue C, Salunkhe SJ, Tomimatsu N, Kawale AS, Kwon Y, Burma S, Sung P, Greene EC: **Bloom helicase mediates formation of large single-stranded DNA loops during DNA end processing**. *Nat Commun* 2022, **13**:2248.

82. Silva GLA, Tosi LRO, McCulloch R, Black JA: **Unpicking the roles of DNA damage protein kinases in trypanosomatids**. *Front Cell Dev Biol* 2021, **9**:636615.

83. Dreesen O, Li B, Cross GA: **Telomere structure and function in trypanosomes: a proposal**. *Nat Rev Microbiol* 2007, **5**:70-75.

84. Jehi SE, Nanavaty V, Li B: ***Trypanosoma brucei* TIF2 and TRF suppress VSG switching using overlapping and independent mechanisms**. *PLoS One* 2016, **11**:e0156746.

85. Jehi SE, Wu F, Li B: ***Trypanosoma brucei* TIF2 suppresses VSG switching by maintaining subtelomere integrity**. *Cell Res* 2014, **24**:870-885.

86. Nanavaty V, Sandhu R, Jehi SE, Pandya UM, Li B: ***Trypanosoma brucei* RAP1 maintains telomere and subtelomere integrity by suppressing TERRA and telomeric RNA:DNA hybrids**. *Nucleic Acids Res* 2017, **45**:5785-5796.

87. Dreesen O, Cross GA: **Telomerase-independent stabilization of short telomeres in *Trypanosoma brucei***. *Mol Cell Biol* 2006, **26**:4911-4919.

88. Dreesen O, Cross GA: **Consequences of telomere shortening at an active VSG expression site in telomerase-deficient *Trypanosoma brucei***. *Eukaryot Cell* 2006, **5**:2114-2119.

89. Hovel-Miner GA, Boothroyd CE, Mugnier M, Dreesen O, Cross GA, Papavasiliou FN: **Telomere length affects the frequency and mechanism of antigenic variation in *Trypanosoma brucei***. *PLoS Pathog* 2012, **8**:e1002900.

90. Glover L, Alsford S, Beattie C, Horn D: **Deletion of a trypanosome telomere leads to loss of silencing and progressive loss of terminal DNA in the absence of cell cycle arrest**. *Nucleic Acids Res* 2007, **35**:872-880.

91. Liu L, Malkova A: **Break-induced replication: unraveling each step**. *Trends Genet* 2022, **38**:752-765.

92. Wu X, Malkova A: **Break-induced replication mechanisms in yeast and mammals**. *Curr Opin Genet Dev* 2021, **71**:163-170.

93. •• Briggs E, Crouch K, Lemgruber L, Hamilton G, Lapsley C, McCulloch R: ***Trypanosoma brucei* ribonuclease H2A is an essential R-loop processing enzyme whose loss causes DNA damage during transcription initiation and antigenic variation**. *Nucleic Acids Res* 2019, **47**:9180-9197.

Demonstration of a role for R-loops in *T. brucei* VSG switching, following from earlier analysis by Briggs et al., 2018, which examined a distinct RNase H enzyme.

94. Briggs E, Crouch K, Lemgruber L, Lapsley C, McCulloch R: **Ribonuclease H1-targeted R-loops in surface antigen gene expression sites can direct trypanosome immune evasion**. *PLoS Genet* 2018, **14**:e1007729.

95. Briggs E, Hamilton G, Crouch K, Lapsley C, McCulloch R: **Genome-wide mapping reveals conserved and diverged R-loop activities in the unusual genetic landscape of the African trypanosome genome**. *Nucleic Acids Res* 2018, **46**:11789-11805.

96. Saha A, Nanavaty VP, Li B: **Telomere and subtelomere R-loops and antigenic variation in trypanosomes**. *J Mol Biol* 2019, **432**:4167-4185.

97. •• Black JA, Crouch K, Lemgruber L, Lapsley C, Dickens N, Tosi LRO, Mottram JC, McCulloch R: ***Trypanosoma brucei* ATR links DNA damage signaling during antigenic variation with regulation of RNA polymerase I-transcribed surface antigens**. *Cell Rep* 2020, **30**:836-851.e835.

First demonstration of a role for ATR kinase signalling of DNA damage in VSG switching and gene expresion control in *T. brucei*, with evidence for an influence on VEX localisation.

98. Chappidi N, Nascakova Z, Boleslavska B, Zellweger R, Isik E, Andrs M, Menon S, Dobrovolna J, Balbo Pogliano C, Matos J, *et al.*: **Fork cleavage-religation cycle and active transcription mediate replication restart after fork stalling at co-transcriptional R-loops**. *Mol Cell* 2020, **77**:528-541.e528.

99. • Matos DA, Zhang JM, Ouyang J, Nguyen HD, Genois MM, Zou L: **ATR protects the genome against R loops through a MUS81-triggered feedback loop**. *Mol Cell* 2020, **77**:514-527.e514.

In this study, the authors uncover a key role for the ATR kinase in tacking R-loops to prevent genome instability. They find that this function of ATR is triggered by the endonuclear MUS81, leading to ATR acting as a restraint to nuclease activty and thereby preventing excessive resection at R-loop sites.

This paper is an example of the many emerging cellular functions of the ATR kinase.

100. Marin PA, Obonaga R, Pavani RS, da Silva MS, de Araujo CB, Lima AA, Avila CC, Cestari I, Machado CR, Elias MC: **ATR kinase is a crucial player mediating the DNA damage response in *Trypanosoma brucei***. *Front Cell Dev Biol* 2020, **8**:602956.

101. • da Silva RB, Bertoldo WDR, Naves LL, de Vito FB, Damasceno JD, Tosi LRO, Machado CR, Pedrosa AL: **Specific human ATR and ATM inhibitors modulate single strand DNA formation in *Leishmania* major exposed to oxidative agent**. *Front Cell Infect Microbiol* 2021, **11**:802613.
Evidence that inhibitors of human ATM and ATR DNA damage signalling kinases are active against *Leishmania*, with potential relevance for *T. brucei* VSG switching.

102. da Silva RB, Machado CR, Rodrigues ARA, Pedrosa AL: **Selective human inhibitors of ATR and ATM render Leishmania major promastigotes sensitive to oxidative damage**. *PLoS One* 2018, **13**:e0205033.

103. Vanhamme L, Poelvoorde P, Pays A, Tebabi P, Van Xong H, Pays E: **Differential RNA elongation controls the variant surface glycoprotein gene expression sites of *Trypanosoma brucei***. *Mol Microbiol* 2000, **36**:328-340.

104. Kassem A, Pays E, Vanhamme L: **Transcription is initiated on silent variant surface glycoprotein expression sites despite monoallelic expression in *Trypanosoma brucei***. *Proc Natl Acad Sci USA* 2014, **111**:8943-8948.

105. •• Faria J, Luzak V, Muller LSM, Brink BG, Hutchinson S, Glover L, Horn D, Siegel TN: **Spatial integration of transcription and splicing in a dedicated compartment sustains monogenic antigen expression in African trypanosomes**. *Nat Microbiol* 2021, **6**:289-300.
In this paper, the authors demonstrate a stable inter-chromosome interaction between the active VSG ES and the splice leader array. Thus, trypanosomes spatially restrict transcription and maturation of RNA to the active locus. The VEX complex facilitate this nuclear organisation, revealing post-transcriptional enhancer-like activity.

106. •• Budzak J, Jones R, Tschudi C, Kolev NG, Rudenko G: **An assembly of nuclear bodies associates with the active VSG expression site in African trypanosomes**. *Nat Commun* 2022, **13**:101.
This study shows that several nuclear bodies associate with the active *VSG* ES, akin to a 'production line' to ensure adequate VSG expression. Furthermore, it is possible that trypanosomes may restrict the number and availablity of nuclear bodies to the active ES to ensure monogenic VSG expression.

107. Chaves I, Zomerdijk J, Dirks-Mulder A, Dirks RW, Raap AK, Borst P: **Subnuclear localization of the active variant surface glycoprotein gene expression site in *Trypanosoma brucei***. *Proc Natl Acad Sci USA* 1998, **95**:12328-12333.

108. Landeira D, Navarro M: **Nuclear repositioning of the VSG promoter during developmental silencing in *Trypanosoma brucei***. *J Cell Biol* 2007, **176**:133-139.

109. Navarro M, Gull K: **A pol I transcriptional body associated with VSG mono-allelic expression in *Trypanosoma brucei***. *Nature* 2001, **414**:759-763.

110. Hughes K, Wand M, Foulston L, Young R, Harley K, Terry S, Ersfeld K, Rudenko G: **A novel ISWI is involved in VSG expression site downregulation in African trypanosomes**. *EMBO J* 2007, **26**:2400-2410.

111. Figueiredo LM, Janzen CJ, Cross GA: **A histone methyltransferase modulates antigenic variation in African trypanosomes**. *PLoS Biol* 2008, **6**:e161.

112. Yang X, Figueiredo LM, Espinal A, Okubo E, Li B: **RAP1 is essential for silencing telomeric variant surface glycoprotein genes in *Trypanosoma brucei***. *Cell* 2009, **137**:99-109.

113. Wang QP, Kawahara T, Horn D: **Histone deacetylases play distinct roles in telomeric VSG expression site silencing in African trypanosomes**. *Mol Microbiol* 2010, **77**:1237-1245.

114. Povelones ML, Gluenz E, Dembek M, Gull K, Rudenko G: **Histone H1 plays a role in heterochromatin formation and VSG expression site silencing in *Trypanosoma brucei***. *PLoS Pathog* 2012, **8**:e1003010.

115. DuBois KN, Alsford S, Holden JM, Buisson J, Swiderski M, Bart JM, Ratushny AV, Wan Y, Bastin P, Barry JD, *et al.*: **NUP-1 Is a large coiled-coil nucleoskeletal protein in trypanosomes with lamin-like functions**. *PLoS Biol* 2012, **10**:e1001287.

116. Maishman L, Obado SO, Alsford S, Bart JM, Chen WM, Ratushny AV, Navarro M, Horn D, Aitchison JD, Chait BT, *et al.*: **Co-dependence between trypanosome nuclear lamina components in nuclear stability and control of gene expression**. *Nucleic Acids Res* 2016, **44**:10554-10570.

117. Padilla-Mejia NE, Koreny L, Holden J, Vancova M, Lukes J, Zoltner M, Field MC: **A hub-and-spoke nuclear lamina architecture in trypanosomes**. *J Cell Sci* 2021, **134**:jcs251264.

118. •• Cestari I, McLeland-Wieser H, Stuart K: **Nuclear phosphatidylinositol 5-phosphatase is essential for allelic exclusion of variant surface glycoprotein genes in trypanosomes**. *Mol Cell Biol* 2019, **39**:e00395-18.
Analysis of the link between inositol phosphate metabolism and monogenic VSG expression in *T. brucei.*

119. Cestari I, Stuart K: **Inositol phosphate pathway controls transcription of telomeric expression sites in trypanosomes**. *Proc Natl Acad Sci USA* 2015, **112**:E2803-2812.

120. York SJ, Armbruster BN, Greenwell P, Petes TD, York JD: **Inositol diphosphate signaling regulates telomere length**. *J Biol Chem* 2005, **280**:4264-4269.

121. • Saha A, Gaurav AK, Pandya UM, Afrin M, Sandhu R, Nanavaty V, Schnur B, Li B: **TbTRF suppresses the TERRA level and regulates the cell cycle-dependent TERRA foci number with a TERRA binding activity in its C-terminal Myb domain**. *Nucleic Acids Res* 2021, **49**:5637-5653.
Demonstration that at least some shelterin components in *T. brucei* interact with RNA as well as DNA.

122. Glover L, Hutchinson S, Alsford S, Horn D: **VEX1 controls the allelic exclusion required for antigenic variation in trypanosomes**. *Proc Natl Acad Sci USA* 2016, **113**:7225-7230.

123. •• Faria J, Glover L, Hutchinson S, Boehm C, Field MC, Horn D: **Monoallelic expression and epigenetic inheritance sustained by a Trypanosoma brucei variant surface glycoprotein exclusion complex**. *Nat Commun* 2019, **10**:3023.
Demonstration that VEX1 (first described by Glover et al., 2016) operates in a complex, which includes a second kinetoplast-specific factor, VEX2.

124. •• Lopez-Escobar L, Hanisch B, Halliday C, Ishii M, Akiyoshi B, Dean S, Sunter JD, Wheeler RJ, Gull K: **Stage-specific transcription activator ESB1 regulates monoallelic antigen expression in *Trypanosoma brucei***. *Nat Microbiol* 2022, **7**:1280-1290.
This study conducted a targeted protein localisation screen of 207 candidates to reveal one protein localising to the ESB, termed ESB1. ESB1 is a stage-specific transcriptional activator that specifically binds the active-VSG ES.

125. Le Noir S, Boyer F, Lecardeur S, Brousse M, Oruc Z, Cook-Moreau J, Denizot Y, Cogne M: **Functional anatomy of the immunoglobulin heavy chain 3 super-enhancer needs not only core enhancer elements but also their unique DNA context**. *Nucleic Acids Res* 2017, **45**:5829-5837.

126. Monahan K, Horta A, Lomvardas S: **LHX2- and LDB1-mediated trans interactions regulate olfactory receptor choice**. *Nature* 2019, **565**:448-453.

127. Sim CK, Perry S, Tharadra SK, Lipsick JS, Ray A: **Epigenetic regulation of olfactory receptor gene expression by the Myb-MuvB/dREAM complex**. *Genes Dev* 2012, **26**:2483-2498.

128. Chaves I, Rudenko G, Dirks-Mulder A, Cross M, Borst P: **Control of variant surface glycoprotein gene-expression sites in *Trypanosoma brucei***. *EMBO J* 1999, **18**:4846-4855.

129. Budzak J, Kerry LE, Aristodemou A, Hall BS, Witmer K, Kushwaha M, Davies C, Povelones ML, McDonald JR, Sur A, *et al.*: **Dynamic colocalization of 2 simultaneously active VSG expression sites within a single expression-site body in *Trypanosoma brucei***. *Proc Natl Acad Sci USA* 2019, **116**:16561-16570.

130. •• Hondele M, Sachdev R, Heinrich S, Wang J, Vallotton P, Fontoura BMA, Weis K: **DEAD-box ATPases are global regulators of phase-separated organelles**. *Nature* 2019, **573**:144-148.
Liquid-liquid phase separation has recently emerged as an important molecular principle of how cells compartmentalise key cellular functions. In this study, the authors show that members of the RNA-dependent DEAD-box ATPases (DDXs) family are global regulators of RNA-containing membraneless organelles, providing cells with spatial and temporal control of various RNA-processing steps, which can ultimately regulate the composition and fate of ribonucleoprotein particles.

131. McLaughlin EJ, Rubio-Pena K, Dujeancourt-Henry A, Glover L: **Transcription dependent loss of an ectopically expressed variant**

surface glycoprotein during antigenic variation in *Trypanosoma brucei*. *mBio* 2022, **13**:e0384721.

132. Lykke-Andersen S, Jensen TH: **Nonsense-mediated mRNA decay: an intricate machinery that shapes transcriptomes**. *Nat Rev Mol Cell Biol* 2015, **16**:665-677.

133. Singh AK, Choudhury SR, De S, Zhang J, Kissane S, Dwivedi V, Ramanathan P, Petric M, Orsini L, Hebenstreit D, *et al.*: **The RNA helicase UPF1 associates with mRNAs co-transcriptionally and is required for the release of mRNAs from gene loci**. *Elife* 2019, **8**:e41444.

134. Delhi P, Queiroz R, Inchaustegui D, Carrington M, Clayton C: **Is there a classical nonsense-mediated decay pathway in trypanosomes?** *PLoS One* 2011, **6**:e25112.

135. Ciaudo C, Bourdet A, Cohen-Tannoudji M, Dietz HC, Rougeulle C, Avner P: **Nuclear mRNA degradation pathway(s) are implicated in Xist regulation and X chromosome inactivation**. *PLoS Genet* 2006, **2**:e94.

136. Marques CA, Tiengwe C, Lemgruber L, Damasceno JD, Scott A, Paape D, Marcello L, McCulloch R: **Diverged composition and regulation of the *Trypanosoma brucei* origin recognition complex that mediates DNA replication initiation**. *Nucleic Acids Res* 2016, **44**:4763-4784.

137. Tiengwe C, Marcello L, Farr H, Dickens N, Kelly S, Swiderski M, Vaughan D, Gull K, Barry JD, Bell SD, *et al.*: **Genome-wide analysis reveals extensive functional interaction between DNA replication initiation and transcription in the genome of *Trypanosoma brucei***. *Cell Rep* 2012, **2**:185-197.

138. Lopez-Farfan D, Bart JM, Rojas-Barros DI, Navarro M: **SUMOylation by the E3 ligase TbSIZ1/PIAS1 positively regulates VSG expression in *Trypanosoma brucei***. *PLoS Pathog* 2014, **10**:e1004545.

139. Narayanan MS, Rudenko G: **TDP1 is an HMG chromatin protein facilitating RNA polymerase I transcription in African trypanosomes**. *Nucleic Acids Res* 2013, **41**:2981-2992.