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Genetic and epigenetic mechanisms underlying cell-surface variability in Protozoa and Fungi

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Abstract (150 words)
Eukaryotic microorganisms have evolved ingenious mechanisms to generate variability at their cell surface, permitting differential adherence, rapid adaptation to changing environments, and evasion of immune surveillance. Fungi such as *Saccharomyces cerevisiae* and the pathogen, *Candida albicans* carry a family of mucin and adhesin genes that allow adhesion to various surfaces and tissues. *Trypanosoma cruzi*, *T. brucei* and *Plasmodium falciparum*, likewise contain large arsenals of different cell surface adhesion genes. In both yeasts and protozoa, silencing and differential expression of the gene family results in surface variability. Here, we discuss unexpected similarities in the structure and genomic location of the cell surface genes, the role of repeated DNA sequences, and the genetic and epigenetic mechanisms -all of which contribute to the remarkable cell surface variability in these highly divergent microbes.

Running title: Genetic and epigenetic mechanisms of cell-surface variability in eukaryotic microbes

Keywords (6)
Phase variation, tandem repeats, satellite repeats, contingency loci, epigenetics, mucin

INTRODUCTION

The outer surface of a microbe is its skin, and like the skin of metazoans it plays a pivotal role in the contact and communication with the environment. Changes in the microbial cell surface can promote adherence to different substrates, alternation between sessile biofilms and motile stages, and evasion of the host's immune system. To effect these changes, the genes encoding the microbial cell surface have evolved to become highly adaptable and flexible. Even isogenic cells within an initially homogeneous clone can generate sub-populations that display differences at their cell surface. How can microbes generate such vast diversity and flexibility from their relatively compact genomes and limited set of genes?

Eukaryotic microbes have evolved elaborate mechanisms to generate cell surface diversity. Variability at the microbial cell surface is driven by multiple mechanisms. Many involve "classic" signaling pathways that sense the environment and in response tune the expression of certain genes. However, other systems generate actual genetic or epigenetic changes that affect expression of specific cell surface genes or even result in the generation of novel genes. Many of these processes may not be tightly regulated, but instead appear to be (partially) stochastic.

Here, we focus on a few of the best-studied examples of cell surface variability in eukaryotes; the yeasts *Saccharomyces cerevisiae* and *Candida albicans* and several protozoans. Although these organisms are quite distant evolutionarily, the comparison reveals a number of common themes. In these microbes the majority of genes encoding cell surface proteins are located in the sub-telomeric regions and in each organism the cell surface genes comprise a gene family that appears to have evolved by gene duplication. These cell surface genes show high frequencies of both genetic and epigenetic changes that confer the protean character to the cell surface. Moreover, many of the proteins encoded by these genes have a similar general structure -an amino terminal leader peptide that guides the protein to the cell surface, a N-terminal variable domain that may have lectin-like binding properties, followed by a variable repeated amino acid motif that may play a role in adherence to host cells and diverse inert surfaces, and a carboxy terminal GPI moiety through which the protein is covalently attached to the exterior of the microbe.

CELL SURFACE VARIATION IN YEASTS

Yeasts are a broad category of unicellular fungi that include harmless saprophytes such as baker' yeast *Saccharomyces cerevisiae* as well as opportunistic pathogens with high mortality rates such as *Candida albicans* and *Candida glabrata*. Yeast cells have adoped a unique survival strategy; they adhere to various substrates and form highly resistant biofilms. These biofilms are complex communities of thousands or even millions of cells. The cells are embedded in a complex extracellular matrix that shields the cells

from chemical insults as well as attacks by the host immune system (for reviews, see (31, 73, 138, 145)).

Adhesion of yeast cells depends on specialized cell surface proteins, called "adhesins". Different types of adhesins confer adhesion to inert substrates (plastic, glass, agar), mammalian tissues and even other yeast cells. The latter type of adhesion is called flocculation, as it results in the formation of large clumps or "flocs" of yeast adhering cells (145)). Adhesins share a common structure (Fig. 1A). The proteins consist of three distinct domains; an N-terminal domain that sometimes shows lectin-like binding to certain sugars or peptides, a large, heavily glycosylated central domain that is believed to confer a rod-like structure to the protein and may also be involved in adhesion, and a C-terminal domain that is responsible for anchoring the protein at the cell surface. The C-terminus carries a glycosylphospatidylinositol (GPI) anchor that anchors the protein in the plasmamembrane (with the N-terminal domain on the outside of the membrane). The GPI anchor is later cleaved off, after which the adhesin is covalently linked to the glucans in the cell wall (14, 51, 67).

The molecular mechanism underlying adhesion is not completely understood. However, it seems that at least two distinct types of mechanisms are at play. First, some adhesins have lectin-like properties and are able to bind sugars or peptides on the surface of other cells (28, 70, 134). A second binding mechanism is less specific and relies on hydrophilic and hydrophobic interactions between the glycosylated central domain and abiotic surfaces such as plastics, glass and metal (58, 66, 82).

Variability in yeast adhesins

It is important for yeast cells to be able to adapt to new conditions and opportunities by adhering to novel substrates or, in the case of pathogenic yeasts, adapting to different host tissues. This variable positive selection drives the formation of novel adhesins, and even though all fungal adhesins share common features, there is a wide variety within the gene family (145, 146). First, different yeast species carry different families of adhesins suited to their specific lifestyle. Second, each yeast cell contains a set of different adhesin genes that provide cells with an array of adhesion properties. The benign brewer's and baker's yeast *Saccharomyces cerevisiae*, for example, possesses a group of adhesin genes, called "*FLO*" (flocculation) genes. The laboratory strains S288C contains five *FLO* genes. *FLO1*, 5, 9 and *10* confer cell-cell adhesion, enabling yeast cells to stick to each other and form large multicellular aggregates (flocculation). Each of the different alleles induces a slightly different level of flocculation: *FLO1* expression results in strong flocculation, while expression of *FLO5*, *9* and *10* confers intermediate or weak clumping (56, 58, 132). By contrast, *FLO11* expression does not result in strong cell-cell adhesion, but rather adhesion to abiotic surfaces such as agar, glass and plastic, as well as the formation of a yeast velum at the liquid-air interface (41, 64, 76, 120). Together, these adhesin genes allow *S. cerevisiae* to adhere to substrates, and form clumps or "flocs" that offer protection from certain stress factors and antifungal drugs (132).

Pathogens such as *Candida albicans* (*ALS* and *EAP* genes) and *Candida glabrata* (*EPA* genes) contain a set of adhesins that allow adherence to (human) tissues and invasive

medical devices such as prostheses, shunts and catheters (28, 63, 81, 137). While the differences in adhesion characteristics of the adhesins in pathogenic fungi have not been characterized in as much detail as those in the ~~benign~~ *S. cerevisiae*, some subtle differences have been reported. Klotz *et al*. found differences in the binding specificity of the *C. albicans* Als1 and Als5 adhesins to various heptapeptides. Moreover, expression of the different *ALS* genes in *S. cerevisiae* results in distinct adhesion to human proteins and cells (129). Similarly, expression of different PA14 binding domains of *C. glabrata* Epa1, 6 and 7 adhesins in *S. cerevisiae* causes adherence to different glycans (32).

Given the evolutionary distance and differences in lifestyles, it is perhaps not surprising that different yeast species carry different adhesins. However, the enormous variety of adhesins between evolutionary closely related species and even strains is remarkable. Research by Hahn and colleagues (59) shows that the *FLO* adhesin gene family in *Saccharomyces* species has evolved and expanded extremely fast. At least two features may be responsible for the quick evolution and expansion of the adhesin family. First, the genes may be under strong and variable selection, which is constantly driving the formation of novel alleles that are better suited to the ever-changing conditions. Second, the fungal adhesins may possess specific properties that make them more susceptible to mutation (114, 144, 146).

Fungal adhesin genes are typically found near the yeast's telomeres. This subtelomeric location plays a pivotal role in the evolution and genetic regulation of the adhesin gene families. First, genes located near the telomeres are subjected to chromatin silencing. In short, the chromatin structure around telomeres fluctuates between "open" and densely packaged "closed" states, which results in more or less stochastic "ON" and "OFF" switching of the local genes. As a result, genetically identical (clonal) populations sometimes show remarkable cell-to-cell variation (so-called "noise") in expression levels of subtelomeric genes (55, 60, 111, 114, 118). Second, (sub)telomeric loci are believed to be subjected to increased recombination frequencies, which could at last partially attributable to their tendency to cluster together (27, 61, 71, 121). Moreover, many adhesin genes contain internal tandem repeats, stretches of DNA that are repeated head-to-tail. The *FLO* genes in *S. cerevisiae*, for example, contain sequences of around 100 nucleotides that are repeated 10-20 times in each gene (144, 146). Such tandem repeats are inherently unstable. The different units within one gene can induce mutation events, mostly by replication slippage. As a result, orthologous and paralogous adhesin genes often vary in length because they contain different numbers of internal repeats (Fig. 1B) (144). The stretch of internal tandem repeats within the adhesin genes leads to a stretch in peptide repeats on the protein level. Because the number of nucleotides within one repeat unit is always a multiple of 3, changing the number of repeats does not alter the reading frame in the downstream part of the adhesin gene, but leads only to a change in the number of repeated peptides within the adhesin (79, 114, 144, 146).

In addition to triggering slippage events that lead to changes in the number of repeats within one adhesin gene, the internal repeats in these genes also trigger recombination events between genes because the sequences of the repeats in different adhesin genes are

often very similar, providing a template for crossing over and homologous recombination (Fig. 1B). Unequal crossing over and other recombination events at the repeat locus can also cause changes in the number of units. Such recombination events could take place between sister chromatids and homologous chromosome pairs. However, the genetic map of *S. cerevisiae* shows that repeats in adhesins form the breakpoints of large segmental duplications near the two telomeres of Chromosome I (adhesin genes *FLO1* and *FLO9*) and the right arm of Chromosome VIII (*FLO5*) (Fig. 1C). This indicates that the repeats within adhesin genes may also trigger recombination events between non-homologous chromosomes (144, 146).

The frequent recombination events within and between adhesins fuel a constantly changing reservoir of different adhesins with slightly different adhesion properties. Changing the number of repeats of the *S. cerevisiae FLO1* adhesin, for example, has been shown to lead to changes in the strength of adhesion. Flo1 alleles with a higher number of internal repeat domains confer strong adhesion, and alleles with less repeats show a gradual decrease in adhesion strength (144). Changes in the *FLO1* repeat region have also been implicated in the binding specificity of the Flo1 protein, with some alleles only binding mannose residues, while others have a broader specificity that includes both mannose and glucose (84). Similarly, changes in the internal tandem repeat of the *FLO11* gene also result in different *FLO11*-dependent phenotypes. Some alleles of *FLO11* encode an adhesin with increased hydrophobic properties that enables cells to form a so-called "flor", a thin biofilm of floating yeast cells at the liquid-air interface (45). Many other fungal adhesins, such as the *C. albicans ALS* genes (99, 115), also contain variable repeats that affect adhesion. Moreover, even cell surface genes in the pathogenic hyphal fungus *Aspergillus fumigatus* have been shown to contain variable internal repeats that may aide to generate variability at the cell surface (80).

Variability in the expression of yeast adhesins

The adhesin genes are induced by several environmental triggers. In *S. cerevisiae*, the *FLO* genes are activated by carbon or nitrogen starvation and changes in ethanol levels (52, 125, 132), as well as quorum sensing agents (23). Inducing adhesins in response to these conditions may allow cells to adapt to stress or forage for nutrients. By contrast, in pathogens, the adhesin genes are induced when cells perceive an opportunity for infection. In *C. glabrata*, for example, the *EPA6* adhesin gene is induced by low levels of nicotinic acid, a precursor of nicotinamide adenine dinucleotide (NAD^+) (40). When the yeast cells are proliferating in blood, where nicotinic acid levels are high, expression of adhesins is repressed. However, in the urinary tract, where nicotinic acid levels are low and the *EPA6* gene is induced, allowing the cells to adhere and colonize the site (40). Similarly, in *C albicans*, several adhesins are induced under low oxygen/low iron conditions encountered in the vaginal mucosal layer (133). Another cue used by *C. albicans* to upregulate its adhesin genes is direct contact with a substrate or matrix (72). An even more direct response between host and yeast cell was described by Prusty and coworkers (110), who showed that the *S. cerevisiae FLO11* adhesin gene is specifically induced by the plant hormone indole acetic acid (auxin). This hormone is abundantly produced at plant wound sites, suggesting that feral yeast strains might perceive this cue as an opportunity for infection.

Whereas yeast adhesins are induced in response to specific conditions and environmental cues, the precise expression patterns show variability between cells. This variability arises because many of the adhesin genes are subjected to epigenetic effects due to the variation in chromatin structure near the telomeres (see above). Expression of subtelomeric fungal adhesins therefore relies on the action of specific chromatin remodeling complexes that help to open the chromatin structure. These processes seem to be a combination of specific, regulated responses to environmental signals and random (stochastic) events. Such stochastic events could explain the observed cell-to-cell variation in a clonal population.

The effect of chromatin-dependent silencing and desilencing has been best characterized for the *S. cerevisiae FLO11* gene. When a population of *S. cerevisiae* cells is placed in conditions that induce *FLO11* expression, some cells in the population will fail to activate *FLO11* expression (60). As a result, a clonal population will contain cells that express *FLO11*, and cells in which *FLO11* is transcriptionally silent. This bimodal expression state of *FLO11* is metastable and inherited from mother to daughter for several generations. However, the expression state is reversible, and some cells do switch states. Relocating the *FLO11* gene to another (non-telomeric) chromosomal site abolishes the variegated expression, indicating that the variegated expression depends on telomere-specific chromatin effects (55). The bimodal expression of *FLO11* relies on several known chromatin remodeling proteins such as Hda1, Rsc1, Gcn5, Snf2 and 5 and Swi1 (6, 46, 60). A similar dependency on certain chromatin remodeling proteins has been demonstrated for several other fungal adhesins, including the *S. cerevisiae FLO10* gene (60) and the *C. glabrata EPA* genes (21, 34, 40).

The cell-to-cell variability of adhesin expression could serve multiple functions. First, the variegated expression may be a bet-hedging strategy. Some cells in the population express adhesins and colonize a surface, whereas other cells do not express adhesins and are therefore able to disseminate in search of new niches. This diversity within the population may also provide cells with a means to act proactively to changing conditions (74). In the case of pathogens, the variegated expression of cell surface proteins may help to elude the host immune system or colonize new tissues.

CELL SURFACE VARIATION IN TRYPANOSOMES

Despite the enormous evolutionary divergence between protozoa and fungi, the trypanosomes *T. cruzi* and *T. brucei* have features that mirror several aspects of the themes displayed by the fungal adhesin gene families, including sub-telomere location, epigenetic silencing, and (in some instances) the structure of the proteins themselves. *T. cruzi* transmitted by reduviid bugs causes Chagas disease, the most important parasitic disease in Latin America, whereas *T. brucei* is transmitted by the tsetse fly and causes African sleeping sickness. *T. cruzi* is an intracellular parasite which is released from infected cells and enters into the bloodstream when cells burst. By contrast, *T. brucei* is

an extracellular parasite carried around in the bloodstream of infected hosts. Both of these parasites, like the fungi, have genetic and epigenetic mechanisms for varying their cell surface adhesion proteins (7).

THE MUCIN FAMILY OF T. CRUZI

The sequencing of *T. cruzi* shows that the diploid genome (22570 predicted proteins) encodes 844 members of the TcMUC sub-family of mucins, cell surface molecules with many similarities to the adhesins of fungi (44). Like the fungal adhesins, the *T. cruzi* mucins are associated with adhesion and attachment to mammalian host cells. Moreover, a subset of mucins shows trans-sialidase activity, an enzymatic activity that allows the parasite to scavenge sialic acid from host cells and incorporate these into its own cell wall. This may not only help with adhering to and entering host cells, it may also promote apoptosis in certain host immune cells and thus function as an immune evasion system (91, 92, 106, 123, 126). Like fungal adhesins, the TcMUC genes are often located in sub-telomeric regions (although some are found in other regions as well) (44). A third similarity between fungal adhesins and *T. cruzi* mucins is the general protein structure: a short hypervariable N-terminal region; a central repeat region containing amino acid residues that are targets for O-linked glycosylation ; and a C terminus containing a GPI anchor sequence (62). The different members of the TcMUC family show strong allelic variation, especially in the number of internal amino acid repeats (3, 20, 44, 62). Moreover, these variable repeat domains may constitute a major fraction of the antigens that are recognized by the host immune system (54).

A striking feature of the *T. cruzi* mucins is that the transition from the insect host to the mammalian host is accompanied by a switch in the mucins that are expressed by the protozoan. In the insect host, the mucin coat is composed of a small subset of relatively homogeneous mucins, whereas in the mammal it is highly heterogeneous (3, 20). Thus, in the insect, the vast majority of mammalian mucins are silenced through some as yet unknown mechanism. The enormous number of different surface mucins displayed in the mammal could mean either that a particular parasite expresses a number of different mucins on its surface or that each parasite expresses a single, but different mucin molecule. *T. cruzi* cells rely heavily on posttranscriptional mechanisms, such as mediation of RNA stability, to regulate gene expression, which makes it more difficult to investigate which mucins are expressed in each cell. If a single cell expresses many mucins, then there may not be epigenetic silencing of the mucin ensemble in the mammalian form. However, if each protozoan in a clone expresses a different mucin gene, then there may be a system akin to that of *T. brucei*, in which only one of the library of mucin genes is expressed. Both of these mechanisms provide a population of parasites that have heterogeneous adherence properties, presumably enabling the organism to adhere to diverse host tissues. Moreover, expression of a large menagerie of cell surface mucins could aid in evasion of the host immune system (1, 20, 105). It is interesting to note that the immune system may also influence which (subsets of) mucins

are observed in the host, as parasites that express certain immunogenic mucins may be weeded out by the immune cells.

THE VARIANT CELL SURFACE PROTEINS OF T. brucei

T. brucei appears to have lost the ensemble of mucin genes present in *T. cruzi*, but encodes another family of cell surface proteins, the Variant Surface Glycoproteins (VSG), which functions like a fur coat, masking other potentially immunogenic cell surface proteins as well as shielding the cell from the complement system. The parasites have developed a remarkable immune evasion strategy using a vast library of VSG genes. Once inside the host, the *T. brucei* cells from the insect host activate the expression of a single member of the large reservoir of VSG genes by an elegant genetic mechanism that constantly switches the sequence of the expressed VSG allele (8, 9, 100-102, 104)

The T. brucei VSG gene family: a mix of divergent genes and pseudogenes

The *T. brucei* genome genome contains at least 1250 – 1500 VSG genes and pseudogenes (13, 140), although the genome sequence is incomplete around telomeric regions, where most of the VSG genes reside. Only 7% of the sequenced genes are intact copies, whereas the majority (66%) are full-length pseudogenes that carry internal stop codons or frameshift mutations. For a detailed view of the (sequenced) VSG genes, refer to the *Trypanosoma brucei* genome DB (www.genedb.org/genedb/tryp) and the VSG database, which provides some tools to search and compare available VSG sequences (www.vsgdb.org) (85).

The *T. brucei* VSG proteins like the mucins of fungi and *T. cruzi* are anchored into the external face of the plasma membrane via a C-terminal glycosylphosphoinositol (GPI) anchor. Mature VSGs contain between 400 and 500 amino acids, with the majority falling between 420 and 460 residues. The proteins are divided into 2 domains, a large hypervariable N-terminal domain of about 350-400 amino acids, and a smaller C-terminal domain of 50-100 residues. Despite their shared structure, the various VSG proteins found in one parasite are surprisingly divergent in their amino acid sequences, with sequence identity falling under 20% for some N-terminal domains (13, 22, 65, 85).

The diversity of the VSG family is extraordinary. Each cell contains an estimated 1250 to 1500 different VSG sequences (13, 140). Moreover, different cells often contain different VSG alleles (140). This enormous diversity originates from frequent recombination events between existing VSG genes that generate novel, chimeric forms of existing VSGs. Apart from generating novel VSG alleles, recombination events in or near VSG genes are also responsible for changes in the VSG allele that is that is transcribed from the active expression site (see next paragraph). VSG switching is relatively frequent, but estimates of exact switching rates vary by several orders of magnitude. Some researchers have reported switching rates between 10^{-6} and 10^{-7} events per cell per generation, which is about 20 fold higher than point mutation rates in *T. brucei* (77, 88), whereas others have observed switching rates as high as 10^{-5}, 10^{-4} and even 10^{-2} events per cellular generation (30, 90, 122, 141).

Frequent recombination events generate novel VSG alleles and cause changes in VSG expression

Only VSG alleles that are present in so-called "expression sites" are transcriptionally active. Despite the existence of multiple expression sites, only one site (and thus one VSG gene) is transcribed at any given time (9, 17, 140). VSG expression sites are located near the telomeres and have a complex structure (Fig. 1A). Apart from a VSG allele, the sites also contain 5 – 10 so-called "expression site-associated genes", as well as one or more so-called "70-bp repeats", tandem arrays of a 70 bp unit (13, 140). The 70-bp repeats, expression site-associated genes, and the VSG allele are all transcribed in one long (40-60 kbp) polycistronic mRNA that is later trans-spliced to yield the individual mature mRNAs.

Since there are only about 45 expression sites in the genome, the vast majority of the more than 1000 VSG alleles reside in transcriptionally silent loci. About half of the SVG genes and pseudogenes reside in tandem arrays of 3 to 250 copies located in the subtelomeric regions. Another 100 to 200 copies lie at the telomeres of the about 100 minichromosomes found in the *T. brucei* genome. In contrast to the subtelomeric clusters located on the large chromosomes, these telomeric minichromosome sites usually contain only one VSG copy and no expression site-associated genes (13, 140).

A switch in VSG expression could result from five basic mechanisms (Fig. 1B) (30, 88, 124, 139, 140). First, duplicative gene conversion can copy a non-active VSG allele into the active expression site, thereby deleting the formerly expressed VSG allele. Similarly, reciprocal gene conversion events can exchange the active VSG allele for a non-active allele (both VSG copies are retained). Third, duplicative telomere conversion can copy a complete telomeric end into the expressed SVG site, thereby deleting the previously active VSG copy. Fourth, reciprocal telomere conversion (also known as telomere exchange) can switch two telomere ends, placing a formerly inactive telomere end in an expressed site, and concurrently moving the formerly active telomere end at the non-active telomere (30, 88, 124, 139, 140). A fifth mechanism of VSG switching that likely does not depend on recombination involves activation of a previously inactive expression site and the concurrent silencing of the previously active site. This process, called "in situ switching" can only draw from a very limited reservoir of VSG alleles, as there are only about 25 metacyclic and 20 bloodstream-stage expression sites.

Some studies have attempted to investigate the relative importance of the various VSG switching mechanisms. In situ switching seems to account for about 16% of all switching events, whereas other events depend on recombination events (30) Surprisingly, however, only a few (<30%) of these recombination events showed a replacement of the active VSG site with a new VSG allele. All other switched mutants seemed to results from complex events that involve both loss of the active site and activation of a new VSG expression site (30, 93, 124).

The molecular mechanisms governing VSG recombination are believed to be similar, if not identical, to mechanisms involved in double-stranded break repair in eukaryotes. The plethora of VSG genes and their surrounding sequences, including the 70 bp arrays, have sufficient similarity to permit homology-based recombination by some sort of double strand break repair. Generation of new VSG's by homology-dependent recombination events is supported by the phenotypes of deletions of some of the genes such as *RAD51* implicated in this type of break repair (26, 87, 109). The majority of VSG rearrangements depend on microhomology between the two sequences that are involved. VSG sequences as short as 11-13 bp., with 1 or 2 mismatches suffice to provide the necessary sequence similarity for recombination. It remains unclear if there is a more specific mechanism or a special fragile site involved in VSG recombination. One likely suspect is the 70 bp repeats that surround most VSG alleles. Such tandem repeats are often hot-spots for recombination, possibly because they form secondary structures that promote DNA polymerase stalling, resulting in DNA breakage (15, 35, 144). However, removing the repeats from the active VSG expression site did not significantly reduce the observed switching rates (88).

Monoallelic expression and in situ switching of VSG genes

How cells silence all but one of the VSG expression sites to establish activity of a single VSG sequence remains a fundamental question. Although some reports indicate that chromatin structure at the telomeric VSG expression sites is unstable, chromatin structure correlates poorly with VSG expression and does not explain why only one allele can be active at a time (96). Hence, it seems unlikely that chromatin silencing is the main mechanism underlying monoallelic VSG expression. Instead, the best clue is that selective expression relies on an unanticipated mechanism involving RNA polymerase I transcription and the position of the VSG expression site within the nucleus.

Unlike most protein coding genes, the VSG genes are transcribed by RNA polymerase (Pol) 1 (57), which usually transcribes ribosomal RNA (rRNA) genes in the nucleolus, sub-compartment of the nucleus. VSG transcription also takes place in a different but dedicated nuclear compartment, called the “ES body” (95). Interestingly, transcription is initiated in multiple, if not all, VSG expression sites, but transcription is aborted in all sites except the single site located in the ES body (143). Deletion of an active VSG expression site leads to rapid activation of a different site, indicating that activation and de-activation are tightly coupled processes (94). Based on these observations, it has been suggested that the ES body can accommodate only one VSG expression site at a time. Hence, VSG switching would rely on one VSG expression site taking substituting for another in the ES body, or one ES body disintegrating while another one is being formed (16, 86, 96, 100) (Fig. 1C).

Despite this progress, many questions about the precise mechanism of VSG switching remain. Which factors present in the ES body allow full transcription of VSG genes? What mediates positioning of a VSG expression site in the ES body? What changes occur during a VSG switching event?

CELL SURFACE VARIATION IN PLASMODIUM FALCIPARUM

P. falciparum, the causative agent of the most severe form of malaria, is responsible for the vast majority of all malaria cases and malaria-related deaths (136). In Africa alone, the annual number of malaria episodes is estimated to be between 10-15 million, resulting in 150,000 to 460,000 annual deaths (29). *Plasmodium spp*. have evolved ingenious mechanisms to vary their cell surface antigens and elude the host's immune response. *P. falciparum*, like yeasts and *T. brucei*, has a reservoir of different surface genes, of which only one or a few are expressed at the same time. However, *Plasmodium falciparum*, unlike Trypanosomes, does not rely on recombination and genomic rearrangements to switch the different *var* genes on and off.

The P. falciparum cell surface genes

The *var* genes, which encode the PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) family of surface antigens are the best studied among the three groups of *P. falciparum* families of variable surface antigens (*var*, *rif* and *stevor (11, 53, 130, 135)*). Each parasite genome contains about 60 different *var* alleles, some of which are clustered at the telomeres, and others in chromosome-central loci. PfEMP1 proteins confer adhesion of infected cells to (healthy) host tissues, blood cells and chondroitin sulphate A, a ubiquitous extracellular glycosaminoglycan (108, 128, 147).

The variable expression of the *P. falciparum var* genes is one of the best documented cases of the direct link between cell surface variation and immune evasion. The evidence shows that the host's immune system specifically responds to the PfEMP antigen, that the different PfEMPs are not always recognized by the same antibodies, and that at any time during malaria infection, there is a complex interplay between the immune response, variant PfEMP expression and the dominant population of parasites. When the immune system produces an antibody that is effective against one PfEMP isoform, rare individuals within the population that produce another var protein proliferate, leaving the immune system one step behind (18, 19, 78, 116, 130).

Structure of the var genes

A *P. falciparum* cell contains a small arsenal of about 60 different *var* genes. Each *var* gene consists of 2 exons. The first exon is extremely variable in both sequence and length (between 3.5 and 9.0 kb) and encodes the two variable extracellular domains of the protein: the Duffy binding like (DBL) and cysteine-rich interdomain (CIDR)) (53). The second exon is between 1.0 and 1.5 kb long and encodes a third conserved cytoplasmic domain containing acidic amino acid residues known as the "ATS" (Acidic Terminal Sequence"). The variable DBL and CIDR motifs confer adhesion to the host's cells (10, 53, 107, 131).

Like the repeated regions of the yeast cell surface proteins the Plasmodium *var* genes contain different numbers of repeats: PrEMP1 variants may have from two to seven DBL

domains and one or two CIDR domains. The variable number of repeats encoding these adhesion domains maybe generated by replication slippage, which by analogy to yeasts may generate diversity in the strength and affinity of adhesion to host cells (39).

Frequent recombination events generate var allele diversity

Different strains often contain very different *var* gene sets, with only minimal overlap between individual genes indicating that there are a lot more different *var* alleles than the 60 variants carried by each strain (49). As is the case for the fungal adhesions, ectopic recombination between *var* genes on different chromosomes occurs relatively frequently. The rearrangements reshuffle *var* fragments to generate novel alleles. In an elegant study, DePristo *et a*l. show that the repeat regions in *var* genes are hot-spots for recombination events in which novel *var* alleles are generated (38). These ectopic recombination events between *va*r genes on different chromosome ends are thought to be facilitated by the telomeric clustering in a "bouquet" structure (33, 50, 61, 139) observed at the nuclear periphery in the sexual parasite form and in the asexual blood-stage (50). As the telomeric and subtelomeric loci in *P. falciparum* are relatively well conserved, each containing similar repeat families and combinations of *var*, *stevor* and *riff* genes (Fig. 2A), there is sufficient sequence similarity to provide the substrates required for recombination. This telomere clustering may be to that observed in yeasts where telomeric chromatin domains often cluster at the nuclear periphery, with several telomeres seemingly aligned and connected in a bouquet structure (27, 33, 97), a configuration that has also been proposed to facilitate (ectopic) recombination (27).

In principle, two major mechanisms could be involved in the ectopic recombination of *var* alleles (Fig. 2B). First, crossover between *var* alleles would result in the reciprocal exchange of a part of each *var* allele. Second, gene conversion could result in the exchange of one *var* fragment by another, but without loss of the fragment in the parental donor allele. Analysis of *var* hybrids produced in 20 different crosses between 2 *P. falciparum* strains showed that in every case, duplicative gene conversion (and not reciprocal exchange) occurred between *var* alleles located on chromosomes 9 and 10, suggesting that gene conversion may be the primary mechanism underlying *var* recombination (50).

Epigenetic silencing restricts var expression to a single gene

Although there are many var genes, the parasites express only a limited subset at any one time (18, 24, 75, 113, 127). Single *P. falciparum* lines have been reported to have switching rates as high as 2% per generation (24, 130). By contrast with *T. brucei*, the switch between surface *var* genes in *P. falciparum* does not appear to involve repositioning of the (active) *var* genes (130). Rather, Plasmodium var genes switch on and off at site without transposing to a new site. Even when multiple *var* transcripts in one cell have been reported, only one *var* gene may be dominant.

The absence of chromosomal rearrangements, and the high switching frequencies observed in monoallelic *var* expression suggest that an epigenetic mechanism may be

involved (127). Silent *var* promoters become active when they are removed from their genomic context and used to drive a reporter gene (36, 37). Such context-dependent silencing could be indicative of a silencing mechanism that depends on chromatin state (55, 118).

A second clue that chromatin remodeling might be involved in *var* silencing and desilencing comes from the observation that the single active *var* allele, or the few simultaneously active alleles, cluster in a distinct perinuclear locus in a perinuclear region of uncondensed euchromatin, whereas silent *var* genes cluster together in tightly packaged heterochromatic regions (42, 43, 112, 148). In addition, silent *var* genes are associated with hypoacetylated histones, whereas active genes are associated with hyperacetylated histones (50) (Fig. 2C). The *P. falciparum* homolog of the yeast silencing Sir2 histone deacetylase, PfSir2, has been shown to bind near (inactive) *var* genes, more specifically at the Rep20 repeat region found near most subtelomeric *var* genes (see Fig. 3A) (42, 50). Deletion of PfSir2 results in desilencing of multiple *var* genes, proving that PfSir2 is nvolved in *var* silencing (42).

The finding that the intron separating in var genes also plays a role in establishing monoallelic expression, adds a new twist to the mechanism. Removing the intron from an active *var* gene causes desilencing of previously inactive *var* promoters. Moreover, the silencing effect of the *var* intron only affects the native *var* promoter (36). A number of observations suggest that internal transcription from the *var* intron may be linked to chromatin-mediated silencing of the var genes (43, 48, 68, 69, 83). However, more direct mechanisms of transcriptional interference cannot be excluded.

Taken together, these observations indicate that phase variation of the *P. falciparum var* genes depends on changes in intranuclear location, chromatin state, and transcription of the *var* introns (Fig. 3C). Among the many questions about this variegation are: First, what is the molecular mechanism that controls switching between the active *var* allele and one of the inactive copies frequency (around 1 switch every 50 generations). It is currently unknown which event happens first –the activation of a *var* gene, or its movement to the euchromatic perinuclear locus (113). Second, how is the telomere clustering related to phase variation? Third, what is the mechanism by which the intron mediates silencing of (neighboring) *var* alleles, and is this mechanism involved in chromatin-mediated silencing.

CONCLUDING REMARKS

The very different microbes discussed in this review have developed genetic mechanisms that generate variability at the cell surface. Whereas the details of each of these mechanism differ, there are several remarkable overall similarities.

A common feature of the mechanism underlying cell surface variability is that cells carry a large reservoir of different cell surface genes of related DNA sequence. However, the size of the gene reservoir differs significantly, ranging from more than 1,000 VSG genes

in *T. brucei* to about 60 *var* genes in *P. falciparum* and 5-20 adhesins in yeasts. In addition to having a large family of related genes, each of the microbes has evolved relatively similar recombination mechanisms to promote the constant generation of novel genes using the old genes as molecular building blocks. The high recombination rates are a consequence of at least 3 factors. First, the relatively high sequence similarity between the different alleles (or certain domains of the alleles) increases the rates of homologous recombination. Second, the location of many genes near telomeres, which may confer unique genetic properties conducive to variability such as elevated mutation and recombination rates. Third, either the DNA surrounding the surface genes (in the case of *T. brucei* and *P. falciparum*), or the surface genes themselves (in the case of fungi and *T. cruzi*) contain repeated sequences that are inherently unstable and may trigger mutation events.

Each of these eukaryotic microbes expresses only one or a few genes of the cell surface gene family at any given time. Genetic and/or epigenetic mechanisms shift the expression from one set of genes to another allowing the cell surface to vary in time. Moreover, cells derived from a single progenitor express different members of the cell surface gene family, so that members of the "clonal population" can have distinct cell surface properties. Some cells may be better adapted to the environment than others, for example when they are able to adhere to particular tissues and/or when they express an epitope to which the host has not (yet) raised an immune response.

A third common principle is the way a limited expression of the library of cell surface genes is established. These organisms rely on chromatin-dependent silencing, possibly in combination with a special nuclear compartmentalization that allows one allele to become active. Other processes affecting the transcription of surface genes may involve transcription from nearby sites, which (directly or indirectly) interferes with transcription of the surface genes. While this mechanism has so far only been described for the *P. falciparum* var intron, it seems that transcriptional interference may also affect yeast adhesin transcription (S. Bumgarner and G.R. Fink, manuscript in preparation).

Although the mechanisms underlying cell surface variability in eukaryotic microbes have some unique features (*e.g.* the importance of telomeres and histones), prokaryotes manifest some of the same common themes behind cell surface variability. For example, the bacterium, *B. hermsii*, like the protozoa, carries multiple genes encoding two different surface antigens, *vlp* and *vsp*. One allele is found in the expression site, whereas all others are silent. Each allele is flanked by short, more conserved sequences, at which sites recombination results in exchange of the active allele (5, 89). In other prokaryotes, unstable DNA regions such as variable tandem repeats or other recombination hot-spots are used to switch expression of surface genes ON and OFF (for an extensive overview of phase variation in prokaryotes, see (142)).

Variation in unstable repeat tracts provides some unique advantages over (point) mutations because they occur at higher frequencies and are completely reversible (a repeat tract can shrink and expand). Hence, unstable tandem repeat tracts may function as evolvable “tuning knobs” that allow swift adaptation to new environments (for a more

elaborate explanation, see (114)). There is evidence that higher organisms also generate variability by repeat recombination. For example, in a remarkable study, Fondon and Garner (47) found that Alx-4 and Runx-2, two genes encoding critical regulators of skeletal morphology contain internal tandem repeats. Just like the coding repeats in fungal adhesins, the number of repeats in these dog genes are variable, and the number of repeats correlates with certain skeletal traits, including the shape of the snout and the number of digits in the pawns. One speculation is that this evolutionary flexibility in skull morphology allows dogs to adapt to new niches and circumstances. The human genome also contains many hundreds of genes containing variable internal tandem repeats (79, 98). The human mucin (MUC) genes, which encode large extracellular proteins similar in structure to the fungal and protozoal mucin genes show considerable size heterogeneity due to the expansion of the internal repeat regions. These size variants have been implicated in cancer and a myriad of other diseases (4, 25). Perhaps the association with abnormal growth is misleading and the variability of these human mucins is required in normal tissue for adaptation to different environments or for the normal course of development .

Summary points (8)

- The microbial cell surface is the interface between the cells and their (variable) environment. As such, the cell surface needs to be sufficiently adjustable and plastic to allow swift adaptation to novel situations. In pathogens, the need for a variable outer surface is even larger because the microbes are under constant attack from the host adaptive immune system.

- Eukaryotic microorganisms have evolved ingenious mechanisms to provide cell surface variability. These mechanisms share common principles, even in evolutionary divergent microbes.

- Surface variability is established by a combination of four major general principles. First, each cell contains an array of different genes of important cell surface genes. Second, only one or a few of these genes are expressed at any given time. Third, expression often switches from one (set of) genes to another. Fourth, the genes have features that increase mutation and recombination events during which novel alleles are being generated.

- Expression of cell surface genes is controlled by a combination of genetic and epigenetic mechanisms. The genetic signaling cascades determine when the genes are expressed. The epigenetic regulation often relies on (more or less stochastic) changes in chromatin state and/or nuclear compartimentalization that silence all but one or a few alleles. The stochastic switches in chromatin structure and/or nuclear localization generate cell surface variability even within clonal populations

- Variable cell surface genes often reside near the telomeres. These loci do not only show high recombination frequencies, but they are also often very susceptible to chromatin-dependent silencing and desilencing.

Future issues (8)

- Which triggers and signal cascades activate and regulate the expression of specific cell surface genes and gene families? Are these potential drug targets?

- How exactly is unitary gen expression of *vsg* and *var* genes established? What is the role of chromatin and nuclear compartimentalization?

- Is cell surface variability constant, or do some mechanisms provide extra (inducible) variability in times of stress? Are such mechanisms active or passive (*e.g.* merely a consequence of increased DNA damage)?

- Do higher eukaryotes employ similar mechanisms to generate variability in other traits and cellular functions?

- Why do telomeric regions show increased recombination rates? Is this only because they tolerate changes more easily (*e.g.* because most of the genes are non-essential, and because gross rearrangements affect less genes when they happen at the chromosome ends), or are there more active mechanisms that induce instability (*e.g.* enzymes, specific chromatin structures or spatial clustering of telomeres in the nucleus)? How is this all affected by aging and stress?

Definitions (10)

Chromatin: Structured complex of DNA and proteins that makes up chromosomes. Changes in the chromatin structure (or "state") affect transcriptional activities of the genes that reside in the locus.

Epigenetic process: Process underlying traits that are inherited from one generation to the next, but that are not encoded in DNA. One common epigenetic mechanism is the inheritance of chromatin states.

Tandem repeat: DNA sequence that is repeated head-to-tail. For example, the DNA sequence "TAGTTAGTTAGTAGTTAGT" is a tandem repeat consisting of 5 units of the sequence "TAGT". Such repeats are inherently unstable during DNA replication, DNA repair, mitosis and meiosis, and as a result, the number of repeated units changes at relatively high frequencies.

Acronyms (10)

VSG : variant Surface Glycoprotein (gene, protein) in *T. brucei*
FLO : Flocculation (gene, protein) in *S. cerevisiae*
GPI : glycosylphospatidylinositol
EPA : EPithelial Adhesin; adhesin (gene, protein) in *C. glabrata*
ALS : Agglutinin-Like Sequence; adhesin (gene, protein) in *C. albicans*
EAP : Enhanced Adherence to Polystyrene; adhesin (gene, protein) in *C. albicans*
SIR : Silent Information Regulator; (gene, protein) involved in chromatin remodeling and silencing.
RAD : RADiation sensitive; (gene, protein) involved in DNA recombination and repair, knockout mutant is sensitive to radiation.

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Figures and Legends

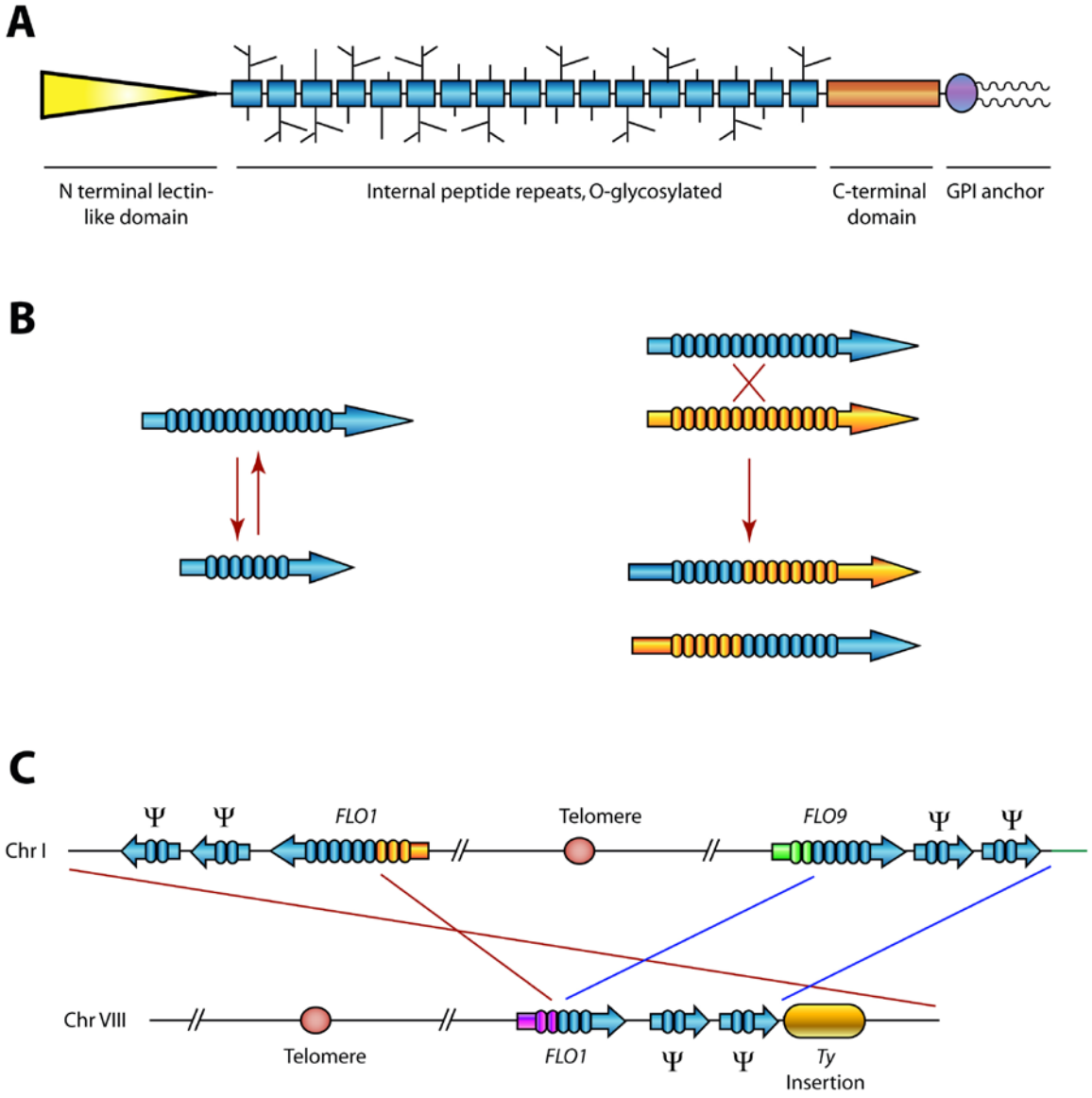

Fig. 1. Variability of fungal adhesins.
A. Typical domain structure of fungal adhesins. The N-terminal domain binds specific peptides or sugar residues. A long middle domain consists of a short (10-50 amino acids) peptide repeat. The repeated structure is often rich in serine and threonine residues which are believed to be heavily glycosylated. The repeat domain confers adhesion to abiotic surfaces and has also been implicated in specific binding to sugars or peptides. The C-terminal domain carries a glycosylphospatidylinositol (GPI) group that transiently anchors the protein in the cell membrane. The GPI anchor is eventually cleaved off and the adhesin is subsequently covalently linked to the outer cell wall structure.

B. The internal tandem repeats in fungal adhesin genes trigger intra- and intergenic recombination events that change the number and/or nature of repeats. The repeated sequences of different adhesins are similar enough to trigger ectopic recombination events, but different enough to confer different types of adhesion. As a consequence, the mutations can change the strength and/or specificity of adhesion and allow fungi to adapt to novel situations and opportunities.

C. The coding tandem repeats in the *S. cerevisiae FLO1*, 5 and 9 genes form the breakpoint of an extensive duplication at both telomeric regions of chromosome 1 and the right arm of chromosome 8, demonstrating that repeats may instigate gross chromosomal rearrangements.

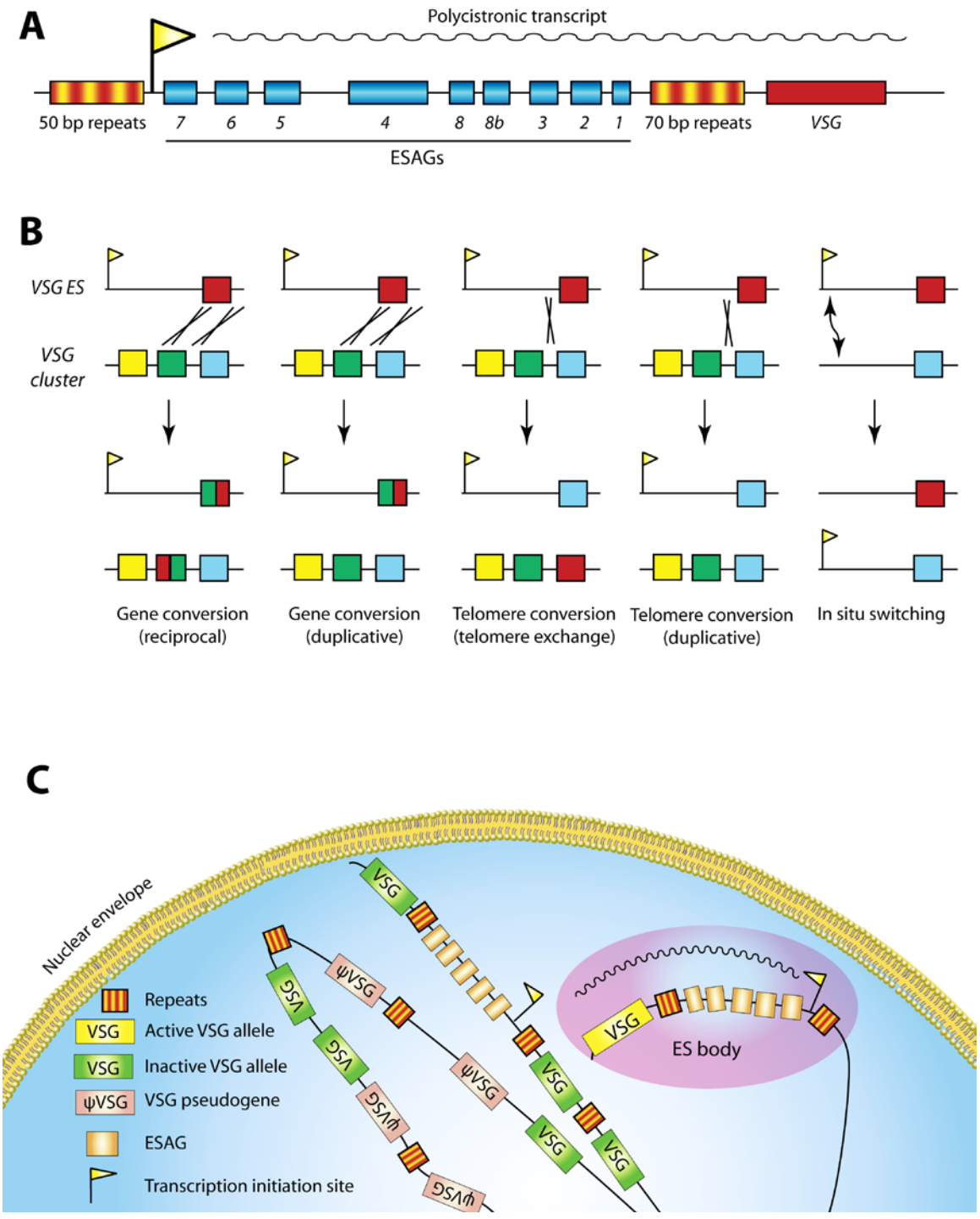

Fig. 2. The Trypanosoma brucei VSG genes.
A. Map of a typical VSG bloodstream-stage expression site. The transcription initiation site (triangle) is located about 40-70 kbp upstream of the (telomeric) VSG gene. Each

site contains a variable set of expression site-associated genes (ESAGs), some of which are associated with adaptation to variable bloodstream environments. There are 11 known ESAG genes, ESAG 1-11, but many of these genes are highly divergent, possibly as a consequence of the high recombination rates associated with VSG sites. The ESAG cluster is flanked by two characteristic tandem repeat sequences, a 50 bp repeat upstream and a 70 bp repeat downstream (2, 12, 103, 117, 119). ***B***. Five possible mechanisms underlying switches in VSG transcription. The first four mechanisms involved DNA recombination between (a part of) an inactive VSG gene or pseudogene and the VSG gene in the expression site. The mechanisms only differ in the number of crossover events and whether the recombination event is duplicative or reciprocal. By contrast, in situ switching does not involve DNA recombination. Here, active expression switches between two distinct VSG expression sites, possibly because a silent site pushes the active expression site out of the Expression Site Body (ES Body) (See text for details). ***C***. Monoallelic expression of VSG genes. Although the genome contains more than a thousand of VSG genes and pseudogenes, only one is expressed at any given time. First, there are only about 20 so-called expression sites (*i.e.* loci with a transcription initiation site). Second, only one such expression site is present in the ES Body where Pol I – dependent transcription elongation is possible. See text for details.

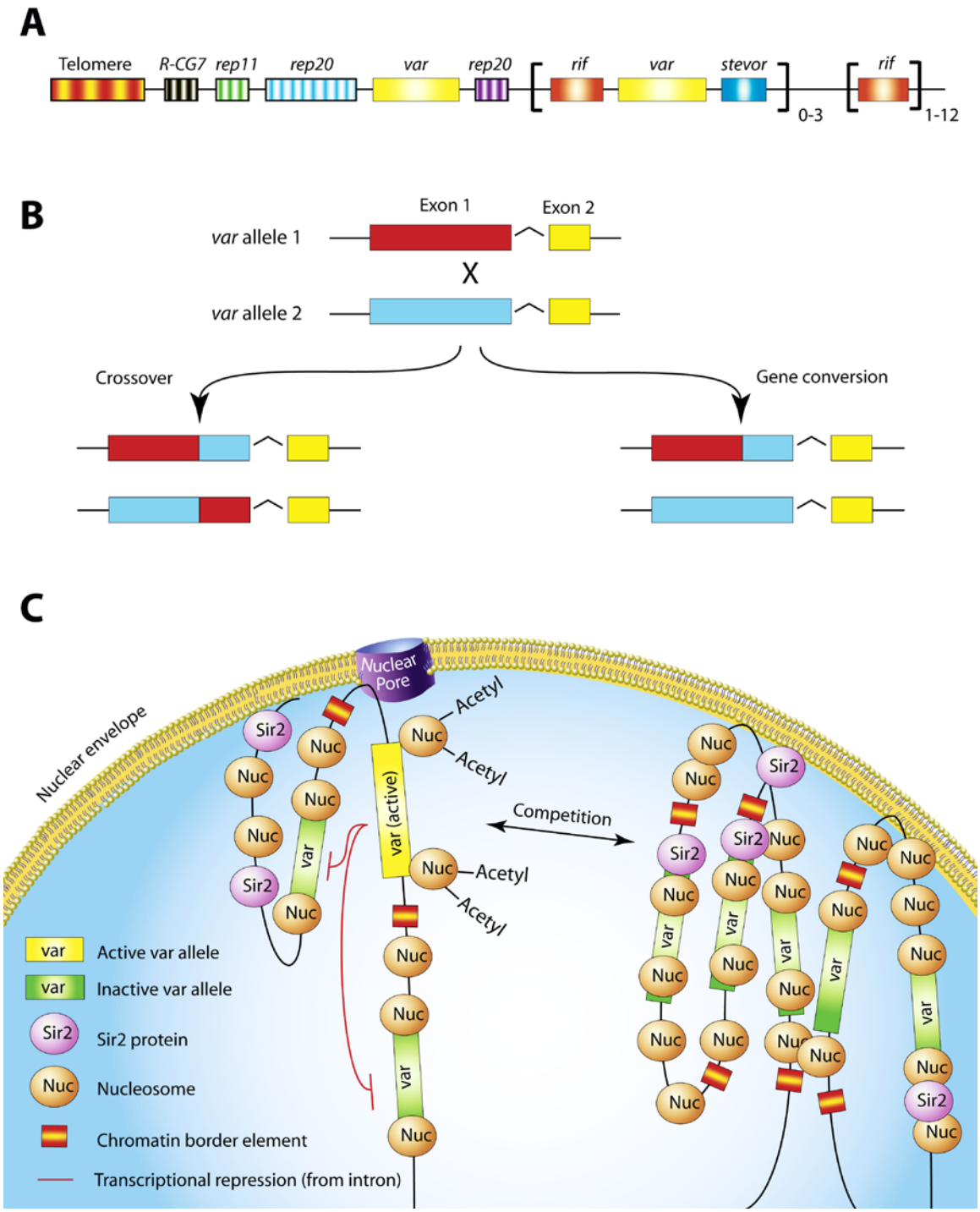

Fig. 3. Phase variation of the Plasmodium falciparum var gene family.
A. Typical subtelomeric organization of three variable surface antigen gene families in *P. falciparum*. Multiple copies of the *var*, *stevor* and *rif* genes are often found clustered together near the telomeres in the *P. falciparum* genome. The genes are interspersed with repetitive elements, including the subtelomeric repeat families *R-CG7*, *rep11* and *rep20*. In addition, some *var* genes are also found clustered together in more central regions of

the chromosomes (not shown). ***B.*** Generation of novel var alleles through crossover or gene conversion. ***C.*** Model for silencing and desilencing of the *var* genes. Most *var* genes are tightly bound by hypo-acetylated histones and are found in condensed, inactive chromatin. By contrast, one or a few *var* genes are part of an active, uncondensed euchromatic nuclear site. Here, most histones are hyperacetylated and only loosely bound to the DNA, allowing expression of the *var* gene, and possibly also transcription of the intron. Activation of the cryptic transcription initation start site in the active var gene's intron may be instrumental in deactivating the other *var* genes. Possibly, this only includes *var* genes on the same *var* cluster and which are also located in the active euchromatic perinuclear site. Through an unknown mechanism, inactive *var* genes can take the place of the active copy at rates of around 2 events per 100 generations (Model based on the model developed by Ralph and Scherf (113)).

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