

# A sirtuin in the African trypanosome is involved in both DNA repair and telomeric gene silencing but is not required for antigenic variation

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## Summary

**Silent information regulator 2 (Sir2)-related proteins or sirtuins function as NAD<sup>+</sup>-dependent deacetylases or ADP ribosylases that target a range of substrates, thereby influencing chromatin structure and a diverse range of other biological functions. Genes encoding three Sir2-related proteins (SIR2rp1–3) have been identified in the parasitic trypanosomatids, early branching protozoa with no previously reported transcriptional silencing machinery. Here we show that, in the mammalian-infective bloodstream-stage of the African trypanosome, *Trypanosoma brucei*, SIR2rp1 localizes to the nucleus while SIR2rp2 and SIR2rp3 are both mitochondrial proteins. The nuclear protein, SIR2rp1, controls DNA repair and repression of RNA polymerase I-mediated expression immediately adjacent to telomeres. Antigenic variation, however, which involves the silencing and Pol I-mediated transcriptional switching of subtelomeric variant surface glycoprotein genes, continues to operate independent of SIR2rp1.**

## Introduction

SIR2-related NAD<sup>+</sup>-dependent (class III) deacetylases or sirtuins (reviewed in Blander and Guarente, 2004) are found in organisms from all biological kingdoms (Frye, 2000). *Saccharomyces cerevisiae* Sir2, the founding member of the group, is a histone deacetylase (reviewed in Rusche *et al.*, 2003) involved in a range of chromatin-mediated processes; namely, gene silencing at telomeres and mating-type loci, DNA repair (Tsukamoto *et al.*, 1997;

Martin *et al.*, 1999), suppression of recombination within ribosomal DNA (rDNA; Gottlieb and Esposito, 1989), DNA replication (Pappas *et al.*, 2004), chromosome stability (Holmes *et al.*, 1997) and plasmid segregation (Longtine *et al.*, 1993). It is now recognized, however, that sirtuins remove acetyl groups from lysines in nuclear, cytosolic and mitochondrial substrates (reviewed in Blander and Guarente, 2004). For example, Sir2 orthologues regulate the function of the p53 transcription factor (Luo *et al.*, 2001; Vaziri *et al.*, 2001) and acetyl CoA synthetase (Starai *et al.*, 2002); and TAF<sub>68</sub> (Muth *et al.*, 2001), tubulin (North *et al.*, 2003) and the archaeal protein, Alba (Bell *et al.*, 2002) are among other known substrates. Sir2 orthologues can also modify small molecules, as in the case of cobB which functions in the cobalamine biosynthetic pathway (Tsang and Escalante-Semerena, 1998), and they have now also been linked to ageing in a range of systems (reviewed in Blander and Guarente, 2004). Thus, sirtuins modulate gene silencing, DNA repair and energy metabolism through post-translational modification of a variety of factors. During the Sir2 deacetylation reaction, acetyl-lysine and NAD<sup>+</sup> are converted to lysine, nicotinamide and O-acetyl-ADP-ribose (Tanner *et al.*, 2000). Thus, the full range of functions assigned to this protein family may be explained by small-molecule substrates and products as well as the array of protein substrates.

*Trypanosoma brucei*, a protozoan that branched early from the eukaryal lineage, is the causative agent of African sleeping sickness. The parasite undergoes antigenic variation in the mammalian host, a process that requires the maintenance of several hundred subtelomeric variant surface glycoprotein (VSG) genes in a transcriptionally silent state (reviewed in Horn and Barry, 2005). Histone acetylation operates in *T. brucei* (Janzen *et al.*, 2006) and one Sir2 orthologue, SIR2rp1, has been characterized in insect stage cells indicating a role in DNA repair through NAD<sup>+</sup>-dependent ADP-ribosylase and deacetylase activities (Garcia-Salcedo *et al.*, 2003). Here, we report characterization of the *T. brucei* sirtuins in bloodstream form cells. In these cells, SIR2rp1 localizes to the nucleus while the other two proteins localize to the parasite's single mitochondrion. Although the nuclear

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protein is involved in telomeric silencing it is not required for VSG gene silencing or antigenic variation.

## Results

### *Three sirtuins expressed in T. brucei*

Three trypanosomatid genomes have been sequenced (see Ivens *et al.*, 2005). Among the sequences, genes encoding three proteins were identified in *T. brucei*, and *Leishmania major* and two in *Trypanosoma cruzi* related to *S. cerevisiae* Sir2. For comparison, the human genome encodes seven Sir2 orthologues and *S. cerevisiae* encodes Sir2 plus four paralogues. The trypanosomatid genes were designated SIR2-related proteins SIR2rp1–3 and it is SIR2rp2 that is not represented in the *T. cruzi* genome (see table S4 in Ivens *et al.*, 2005). Phylogenetic analysis places SIR2rp1 in a group with ScSir2, HsSIRT1 and HsSIRT2, while SIR2rp2 and SIR2rp3 are more closely related to bacterial proteins and HsSIRT4 and HsSIRT5 respectively (Fig. 1A). Although all trypanosomatid SIR2rp's lack the N-terminal extension of ScSir2 that is required for nucleolar localization (Cockell *et al.*, 2000), they all contain a full catalytic domain (*T. brucei* proteins illustrated in Fig. 1B). The SIR2rp1 proteins have Ser-rich motifs towards the C-terminus and both SIR2rp2 proteins contain two insertions, the larger of which aligns with an insertion in the HsSIRT4 and the *Streptomyces coelicolor* proteins (see Fig. 1). All eight trypanosomatid proteins have a Zn<sup>2+</sup> binding motif of the CX<sub>2</sub>CX<sub>20</sub>CX<sub>2</sub>C type but one of the Cys residues is absent in SIR2rp3 in all three trypanosomatids (see *T. brucei* proteins aligned in Fig. 1C), a feature shared with the HsSIRT5 and the *Salmonella enterica* proteins (see Fig. 1A). These findings indicate that each of the three SIR2rp's has similar features regardless of the trypanosomatid under consideration. We have focused on the *T. brucei* SIR2rp's found on chromosomes VII, VIII and IV encoding proteins with predicted molecular mass of approximately 38.5, 33.6 and 27 kDa respectively.

We sequenced all three *T. brucei* genes from the Lister 427 strain and compared the sequences to the *T. brucei* genome reference strain (TREU 927/4). Each predicted protein differed at only two positions as follows; SIR2rp1, I161M and S281N; SIR2rp2, Q61R and D304G; SIR2rp3, R6G and D132E. All mature *T. brucei* mRNAs possess an identical 5'-spliced leader (SL) sequence added at a specific AG-dinucleotide, splice acceptor (SA). Using reverse transcription polymerase chain reaction (RT-PCR), we confirmed that all three genes are expressed in bloodstream form cells (data not shown) and identified *trans*-splicing sites, 198, 115/67 and 23 nt upstream of the start codon for

SIR2rp1–3 respectively. We also detected SIR2rp1 mRNA by Northern blot analysis using polyA<sup>+</sup> mRNA, revealing a transcript of approximately 1.8 kb in both mammalian bloodstream and insect mid-gut life cycle stages (data not shown).

### *SIR2RP subcellular localization*

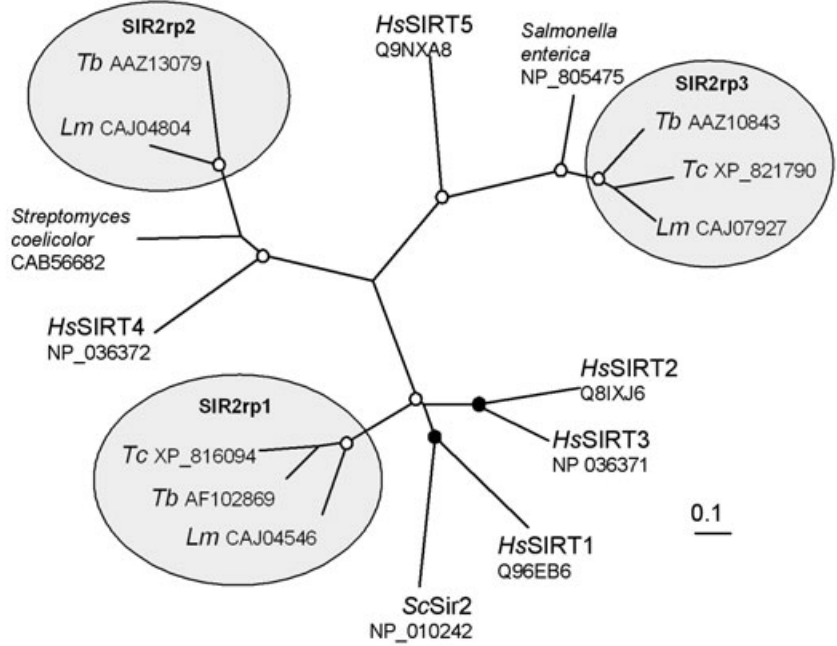
To gain insight into the *T. brucei* cellular compartment where each protein acts, we expressed inducible, tagged (cMyc or eGFP) versions of each protein in bloodstream form cells. In the absence of Tet, N-terminal cMyc-tagged mycSIR2rp1 was expressed at a low-level while Tet increased expression significantly (Fig. 2A). We found that mycSIR2rp1 induction was toxic causing a significant decrease in cell growth within 48 h (Fig. 2B). SIR2rp1 was previously reported to be a nuclear protein in insect-stage cells (Garcia-Salcedo *et al.*, 2003). Analysis of fixed cells indicated a weak, but specific signal throughout the nucleus when mycSIR2rp1 was expressed at a low level, while increased expression caused the protein to accumulate in the cytoplasm (Fig. 2C). Other cultures did not display growth defects following Tet addition (data not shown) indicating that it is increased mycSIR2rp1 expression and/or accumulation in the cytoplasm that is toxic.

Phylogenetic analysis indicated that trypanosomatid SIR2rp2 and SIR2rp3 are related to bacterial proteins and, respectively, the human mitochondrial proteins, HsSIRT4 and HsSIRT5 (Fig. 1A and see Michishita *et al.*, 2005). Putative N-terminal signal sequences are indicated in LmSIR2rp2 and TbSIR2rp3 in the genome sequence database (<http://www.genedb.org/>). We therefore cloned SIR2rp2 and SIR2rp3 genes in a C-terminal GFP-tagging vector. Analysis of fixed cells expressing either SIR2rp2<sup>GFP</sup> or SIR2rp3<sup>GFP</sup> revealed GFP signals reminiscent of the single mitochondrion found in trypanosomes. We costained these cells with Mitotracker Red and merged images revealed almost total colocalization, indicating that both SIR2rp2 and SIR2rp3 are indeed mitochondrial proteins (Fig. 3B). When we expressed either of these proteins with an N-terminal tag we observed cytoplasmic localization of the full-length protein (data not shown) consistent with the idea that these tags interfere with N-terminal mitochondrial import signals.

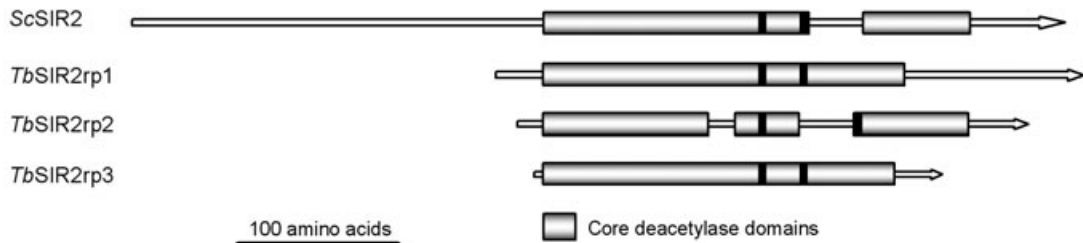
### *SIR2rp1 influences DNA repair in bloodstream form cells*

SIR2rp1 is the only nuclear sirtuin in *T. brucei* (Fig. 2, and also see Fig. 3). To explore the role of this protein, we targeted the cognate gene for disruption in bloodstream form *T. brucei* cells. The SIR2rp1 gene is a 'single copy'

**A**



**B**



**C**

ScSIR2	256	RKILVLTGAGVSTSLGIPDFRS-SEG	FYSKIKHLGLDDEQDVENYNIFMHP	PSVFYNIAN	314
TbSIR2rp1	30	TKLIFVMVGAGISVAAGIPDFRS	PHTGLYAKLSRYNLNSPEDAFSL	PLLRQOPSVFYNIILM	59
TbSIR2rp2	15	QRCVILTGAGCSTESGVDPYRG-PNGL	YRRPNFVPLTR---QVFLSGSEHQ	KRYWARSMF	70
TbSIR2rp3	6	RMLAILTGAGISAESGISTFRD-QNGL	WENHRVEDVCTEAAFLKQP	TVVQR---IYNNRR	61
▼					
ScSIR2	315	--MVLPPKEKIYSP	LHSFIKMLQMKG--KLLRNYTONIDN	LESYAGIS	TDKLVQC 364
TbSIR2rp1	60	DMDLWPGKYCPTTVH	HFISLLAKKG--MLLCCCTONIDGLER	ACGIP	ESLLVEA 141
TbSIR2rp2	71	GYNTVSGASCN-DTHM	GLYELRYAG--VVNRLLTQNV	DLGHHLAHG -16-	NSGVLEL 137
TbSIR2rp3	62	R-ALLSPEVKPNASH	QALARLQREYKDGQVVIITQ	NIDDLHERAGSR	--QVLHM 112
▼					
ScSIR2	365	HGSEFATATCVTCHWN	LEGERIFNKIRNLELPL	GPYCYKK -33-	GVLKPDITF 445
TbSIR2rp1	142	HGSESSASCVDCHAK	YDINIARAETRAGKVEH	CNQC	GIVKPDVVF 187
TbSIR2rp2	138	HGNIHQVCCMOCGDV	SPRRRLQORLCEANYQL -31-	CEHCG--	GLLKPVVV 214
TbSIR2rp3	113	HGELLKVRCTATGRV	FESRDDVIHGSEK---	CECCGVV	ETLRPHIVV 156
▼					
ScSIR2	446	FGALPNKEHKSIRE	DILECDLLICIGTSLKVPVSEI	VNMVPSH-VEQV	LINRDPV 501
TbSIR2rp1	188	FGENLPEAFN-VAGL	IEBELLLILGTSLOVHPFADL	LALMVPSD-VERV	LFNLERV 242
TbSIR2rp2	215	FGENVKCEVREAYT	AVRAASCLICIGTSLQVFSAL	REVLAARESGV	EIAIVTAGRT 271
TbSIR2rp3	157	FNEMP--LYMDV	IDEVVQNAGLFVAVGTSGNV	YPAGLVMI	AKAHSAGTLELNLEFS 211

**Fig. 1.** Sirtuins in trypanosomatids.

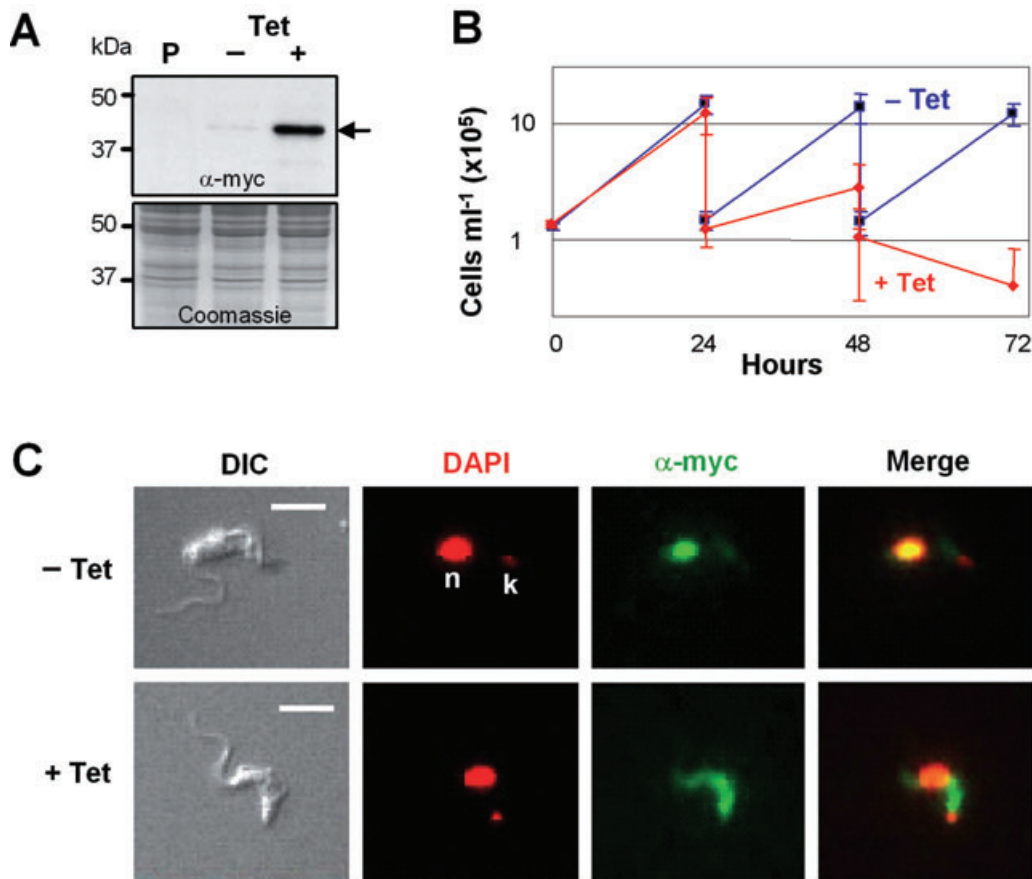
A. The trypanosomatid SIR2rp's were compared to other Sir2 orthologues identified by BLAST analysis. The unrooted neighbour-joining tree was generated using Clustal 1.8 × and TreeView. Branching confidence based on bootstrap replicas is indicated where excellent (> 99%, open circle) or very good (> 90%, closed circles). Trypanosomatid SIR2rp's are indicated in shaded ovals. *Tb*, *T. brucei*; *Tc*, *T. cruzi*; *Lm*, *L. major*; *Hs*, *Homo Sapien*; *Sc*, *S. cerevisiae*.

B. Schematic representation of the predicted *T. brucei* proteins compared with *S. cerevisiae* Sir2. The location of Cys residues that form the Zn<sup>2+</sup>-binding motif are indicated (black bars).

C. The core deacetylase domains from the *T. brucei* SIR2rp's were aligned with the equivalent region from *Sc*Sir2. Residues that are identical between *Sc*Sir2 and any of the *T. brucei* proteins are white on a black background. Other residues shared among the *T. brucei* proteins are on a grey background. Asterisks indicate a putative Zn<sup>2+</sup>-binding motif. Bars above the sequence indicate the location of conserved motifs used to design PCR primers (see *Experimental procedures*). Arrows and arrowheads indicate critical catalytic or NAD<sup>+</sup>-binding residues respectively (see Imai *et al.*, 2000; Min *et al.*, 2001; Chang *et al.*, 2002).

gene and *T. brucei* is diploid so we assembled a pair of constructs with different selectable markers. The constructs were used to disrupt residues 1–285 (see Fig. 4A). PCR (data not shown) and Southern analysis (Fig. 4A) indicated that the gene was disrupted as intended. Features that were indistinguishable between wild type and *sir2rp1* null mutants (data not shown) include growth rate in

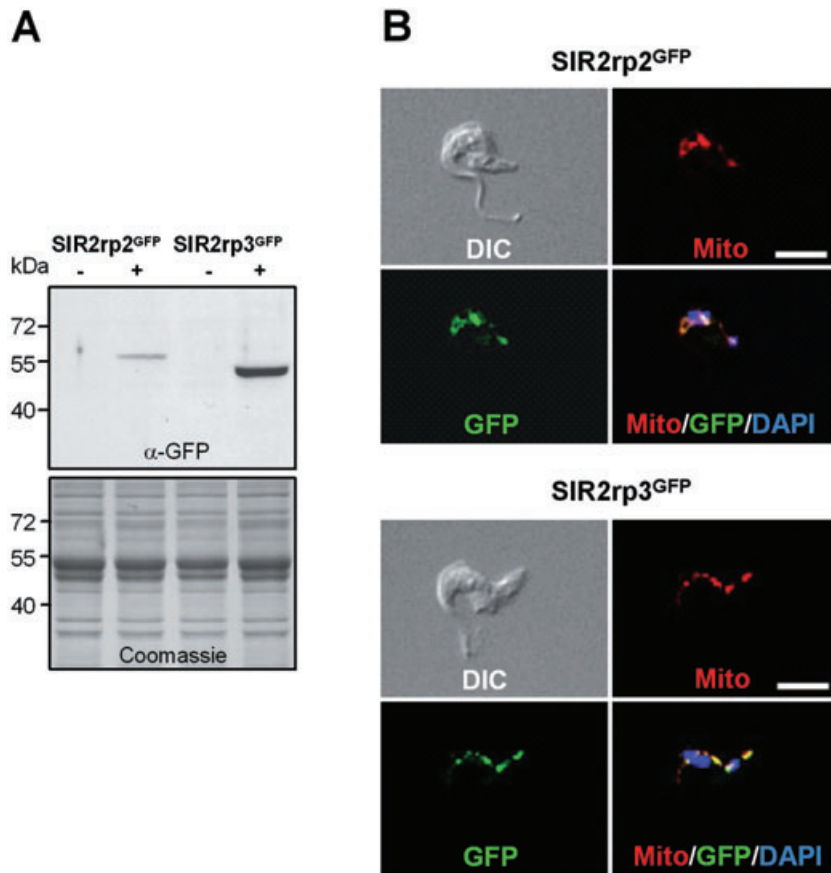
culture, differentiation to the insect-stage with concomitant inactivation of *VSG* expression and tubulin acetylation status, as assessed using C3B9 antibodies (Sasse and Gull, 1988) on Western blots. We also disrupted the *SIR2rp2* and *SIR2rp3* genes. These mutant strains displayed no growth or differentiation defects (data not shown).

**Fig. 2.** SIR2rp1 localizes to the nucleus.

A. Western blotting with anti-cMyc and cell-lysates from parental (P), and a representative <sup>myc</sup>SIR2rp1 cell line before (–) and after induction (+; 1 μg ml<sup>-1</sup> tetracycline for 24 h). The arrow indicates <sup>myc</sup>SIR2rp1 which is expressed at a low level prior to induction. Bottom panel: Coomassie-stained gel as a loading control.

B. Growth curves generated from four independent <sup>myc</sup>SIR2rp1 cell lines in the presence or absence of Tet. Cultures were diluted to 10<sup>5</sup> cells per ml as appropriate every 24 h. Error bars: one standard deviation.

C. Immunofluorescence detection of <sup>myc</sup>SIR2rp1 (green). <sup>myc</sup>SIR2rp1 expressed at a low level localizes to the nucleus (upper panels, detected by long exposure) or the cytoplasm when expression level increases (lower panels). DNA counterstained with DAPI (false coloured in red); k, kinetoplast; n, nucleus. Scale bar: 5 μm.



**Fig. 3.** SIR2rp2 and SIR2rp3 are mitochondrial proteins.

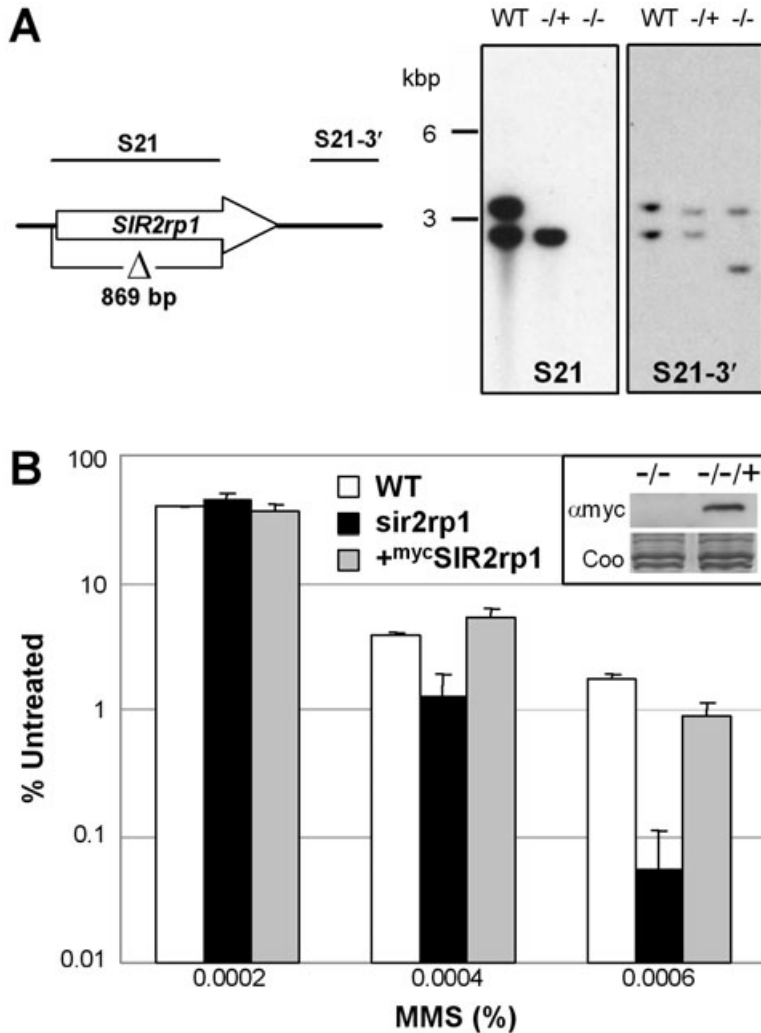
A. Western blotting with anti-GFP and cell-lysates from representative SIR2rp2<sup>GFP</sup> and SIR2rp3<sup>GFP</sup> cell lines before (–) and after induction (+; 1 µg ml<sup>-1</sup> tetracycline for 24 h). Bottom panel: Coomassie-stained gel as a loading control.

B. Immunofluorescence detection of GFP (green) in fixed cells revealed mitochondrial localization of SIR2rp2<sup>GFP</sup> and SIR2rp3<sup>GFP</sup> MitoTracker CMXRos (Mito) labelling (red). DNA counterstained with DAPI (blue). Scale bars: 5 µm.

*Kluyveromyces lactis sir2* mutants display hypersensitivity to DNA targeting drugs (Chen and Clark Walker, 1994) and assays with the DNA-damaging agent, methanesulphonic acid methyl ester (MMS) indicated that SIR2rp1 is involved in DNA repair in insect stage *T. brucei* (Garcia-Salcedo *et al.*, 2003). We first confirmed a role for SIR2rp1 in DNA repair in insect stage *sir2rp1* null mutants (data not shown) and then applied the MMS assay to bloodstream form cells where the IC<sub>50</sub> is about 10-fold lower than for the insect stage (see Proudfoot and McCulloch, 2005). Bloodstream form *sir2rp1* null mutants were more susceptible to MMS than wild-type cells (Fig. 4B). To confirm that increased MMS-sensitivity was a consequence of SIR2rp1 deficiency, *sir2rp1* cells were reconstituted with an expression vector which restored SIR2rp1 expression (Fig. 4B, inset). This was achieved by targeting a constitutively expressed *myc*SIR2RP1 gene to different rDNA spacer loci which support distinct expression levels (see Alford *et al.*, 2005). Only strains expressing SIR2rp1 at a sub-toxic level (see Fig. 2) could be generated using this strategy. Restored SIR2rp1 expression reversed the increased MMS-hypersensitivity of *sir2rp1* null mutants (Fig. 4B) indicating a role in DNA repair in both major life cycle stages of *T. brucei*.

#### *SIR2rp1* influences telomeric silencing but is not required for antigenic variation

Telomeric gene silencing has been demonstrated in mammalian cells, in yeast, *Plasmodium falciparum* and *T. brucei* (see Glover and Horn, 2006), and silencing is known to rely upon Sir2 in yeast (reviewed in Rusche *et al.*, 2003) and a sirtuin in *P. falciparum* (reviewed in Figueiredo and Scherf, 2005). In addition, *T. brucei* SIR2rp1 was reported previously to be associated with telomeric DNA (Garcia-Salcedo *et al.*, 2003). We first tested SIR2rp1 in a telomeric silencing assay in *S. cerevisiae*. SIR2rp1 failed to complement a *Scsir2* defect (strain UCC2, data not shown) but overexpression of SIR2rp1 and SIR2rp3 disrupted silencing (Fig. 5). This dominant negative effect, which is similar to that observed for the yeast cytosolic Sir2 paralogue, HST2 (Perrod *et al.*, 2001), could reflect competition with Sir2 for a substrate or ligand, such as NAD<sup>+</sup>, or production of nicotinamide, a Sir2 inhibitor (Bitterman *et al.*, 2002). One possible explanation for a lack of activity for SIR2rp2 may be a requirement for mitochondrial import and the associated processing as is the case with human SIRT3 (Schwer *et al.*, 2002). Although the yeast analysis demonstrated a clear activity for SIR2rp1, it did not provide



**Fig. 4.** SIR2rp1 controls DNA repair in bloodstream form *T. brucei*.

**A.** Schematic maps describing the gene disruption strategy (left-hand side).  $\Delta$  indicates the region targeted for deletion. Probes used in Southern analysis are illustrated above the map. Gene disruption was verified by Southern hybridization (right-hand side). Genomic DNA from wild type and transformed strains was digested with KpnI. Both *SIR2rp1* alleles are detected due to the presence of a polymorphic KpnI site at this locus.

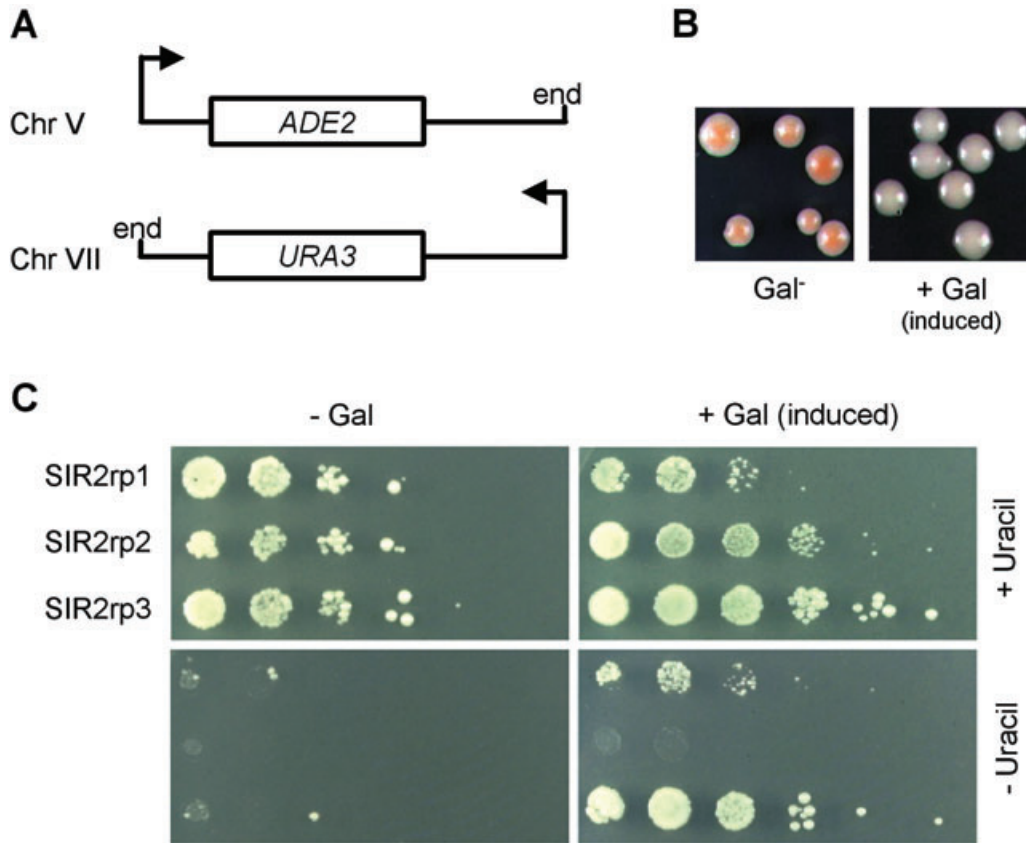
**B.** DNA repair assay. Cultures were seeded at  $10^5$  per ml in triplicate in the presence of methanesulphonic acid methyl ester (MMS) at the concentration indicated and counts were taken after 32 h. Error bars: standard error. The inset (upper panel) shows  $\alpha$ mycSIR2rp1 expression (-/-/+) in cells with both native *SIR2rp1* alleles disrupted (-/-). Coo, Coomassie-stained loading control (lower panel).

evidence for a direct role in telomeric silencing. We therefore turned to analysis in *T. brucei*.

In *T. brucei*, telomeric silencing interferes with RNA polymerase I (Pol I) transcription (Glover and Horn, 2006) so we monitored the expression of telomere-adjacent Pol I-driven reporter cassettes in *sir2rp1* null mutants. Consistent with a role in telomeric silencing, in cells with a repressed promoter 2 kb from a telomere (Fig. 6A), reporter expression was increased following *SIR2rp1* disruption (Fig. 6B). Northern blot analysis indicated a sixfold increase in reporter expression in *sir2rp1* null mutants. In addition, *sir2rp1* cells reconstituted with SIR2rp1 (Fig. 4B, inset) displayed restored repression (Fig. 6B) confirming that loss of silencing was a consequence of SIR2rp1 deficiency. Tubulin, a Pol II transcript derived from a telomere-distal gene array, appeared unaffected by SIR2rp1. Analysis of differentiated, insect-stage cells gave similar results (data not shown). Thus, our results demonstrate a role for SIR2rp1 in telomeric silencing in both major life-cycle stages. Telomeric silencing only

spreads a short distance from the telomere at an *RRNA* locus (Glover and Horn, 2006) so we considered the idea that increased SIR2rp1 expression could extend the repressed domain. Inducible overexpression of  $\alpha$ mycSIR2rp1 failed to 'spread' silencing to a promoter 5 kb from an ectopic telomere at an *RRNA* locus (data not shown). In fact, it is Sir3, not Sir2, that is the limiting factor for the spread of silencing in *S. cerevisiae* (Hecht *et al.*, 1996). Sir3 orthologues are not found beyond yeasts but our results are consistent with accessory factor-dependent restriction and/or spreading in *T. brucei*.

In *T. brucei*, 'basal' telomeric gene-silencing operates in both major life cycle stages. A second form of repression specifically associated with *VSG* gene expression sites operates only in bloodstream-form cells (Glover and Horn, 2006). The data above indicate that basal telomeric silencing is SIR2rp1-dependent. To assess the influence of SIR2rp1 on *VSG*-associated repression we engineered Pol I reporter cassettes upstream of a *VSG221* gene (Fig. 6C) in cells with *VSG221* either expressed or



**Fig. 5.** SIR2rp1 disrupts telomeric silencing in *S. cerevisiae*.

A. The UCC3505 strain has an intact *ScSIR2* gene and repressed *ADE2* and *URA3* reporters adjacent to telomeres on chromosomes V and VII respectively.

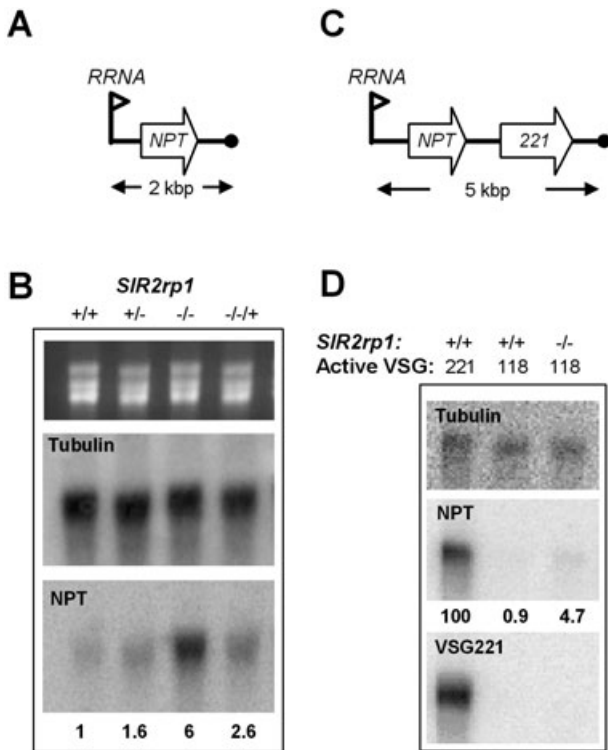
B. *ADE2* assay. Colonies are red when *ADE2* is silenced and white when *ADE2* is expressed so the emergence of white colonies on galactose plates indicates that the telomeric *ADE2* gene was derepressed by SIR2rp1.

C. *URA3* quantitative growth assay. Ten-fold serial dilutions. Because the telomeric *URA3* gene is silenced the strain is auxotrophic for uracil (left-hand panels). SIR2rp1 expression allowed growth on plates lacking uracil confirming that SIR2rp1 disrupts telomeric silencing (right-hand panels).

repressed. The latter cells expressed *VSG118* from a telomeric expression site on another chromosome and cells expressing *VSG221* acted as a positive control for reporter expression. *SIR2rp1* was disrupted in *VSG118*-expressing cells and the desired disruption was confirmed by Southern blotting (data not shown). The Pol I reporter was partially (fivefold) de-repressed in *sir2rp1* null mutants, as determined by northern analysis, but mRNA from the downstream *VSG221* gene remained undetectable indicating strong residual repression at this locus (Fig. 6D). Thus, the SIR2rp1 effect is detected 5 kb from the telomere at the *VSG* locus but a SIR2rp1-independent mechanism maintains *VSG* gene silencing even when an ectopic promoter is placed just upstream. We reasoned that if *VSGs* were de-repressed to a sufficient level, mice would mount an immune response and eliminate the parasites after the first 'wave' of parasitaemia. We found no evidence for *VSG* derepression in mice, however, because the *sir2rp1* null mutants established infection

and, similar to wild-type cells, generated two waves of parasitaemia peaking 6 and 12 days following inoculation (data not shown).

The data above indicate maintained *VSG* repression in the absence of SIR2rp1. We finally assessed the ability of *sir2rp1* null mutants to undergo antigenic variation and to establish *VSG* repression. Switching from *VSG118* to *VSG221* expression was previously shown to occur at a frequency of  $\sim 6 \times 10^{-7}$  (Horn and Cross, 1997a) via one of two distinct mechanisms (see Fig. 7A). For these experiments, we used strains expressing *VSG118* with an *NPT* reporter upstream of the repressed *VSG221* gene and an equivalent *sir2rp1* null mutant strain (see Fig. 6). To compare switching frequency in the two strains, we seeded 10 independent cultures with  $\sim 10$  cells from each and, when the cultures had expanded to  $1\text{--}2 \times 10^6$  cells, added G418 at  $200 \mu\text{g ml}^{-1}$ . The vast majority of cells were rapidly eliminated under these conditions and, importantly, at least one culture from each strain did not



**Fig. 6.** SIR2rp1 controls telomeric gene silencing. A. An *NPT* gene was placed 2 kb from a *de novo* telomere (see Glover and Horn, 2006) in strains with different SIR2rp1 genotypes: +/+, wild type; +/-, heterozygous; -/-, disrupted; -/-+, complemented with *myc*SIR2rp1 (see Fig. 4). B. *NPT*<sup>2</sup> reporter expression was assessed by Northern blotting in bloodstream form cells. Numbers indicate normalized *NPT* expression as assessed by Phosphorimager analysis (Amersham). C. An *NPT* gene was placed 5 kb from a telomere within the polycistronic *VSG221* expression site followed by *SIR2rp1* disruption (see Fig. 4A). D. *NPT*<sup>VSG-5</sup> and *VSG221* expression were assessed as in B.

recover confirming that the *NPT* reporter remained repressed in most cells (see Fig. 6D). After approximately 1 week, a number of G418 resistant cultures emerged. To determine whether these cultures had coactivated *VSG221*, we made whole-cell protein extracts and separated proteins in SDS-PAGE gels. Coomassie-staining confirmed a *VSG* switch in nine of the *SIR2rp1* wild-type cultures (from a total of  $\sim 1.8 \times 10^7$  cells) and in four of the *sir2rp1* null mutant cultures (from a total of  $\sim 1.1 \times 10^7$  cells). Two switches from each strain are shown in Fig. 7B. Although some of the wild-type cultures may not have been clonal, this equates to switching frequencies of  $\sim 5 \times 10^{-7}$  and  $\sim 3.6 \times 10^{-7}$  respectively. SIR2rp1 therefore has no significant impact on *VSG* switching frequency in these cells.

To determine whether *sir2rp1* null mutants could establish *VSG* repression we needed to look for *VSG221*-expressing cultures that retained the *VSG118* gene. Southern blotting indicated that the *VSG118* gene could

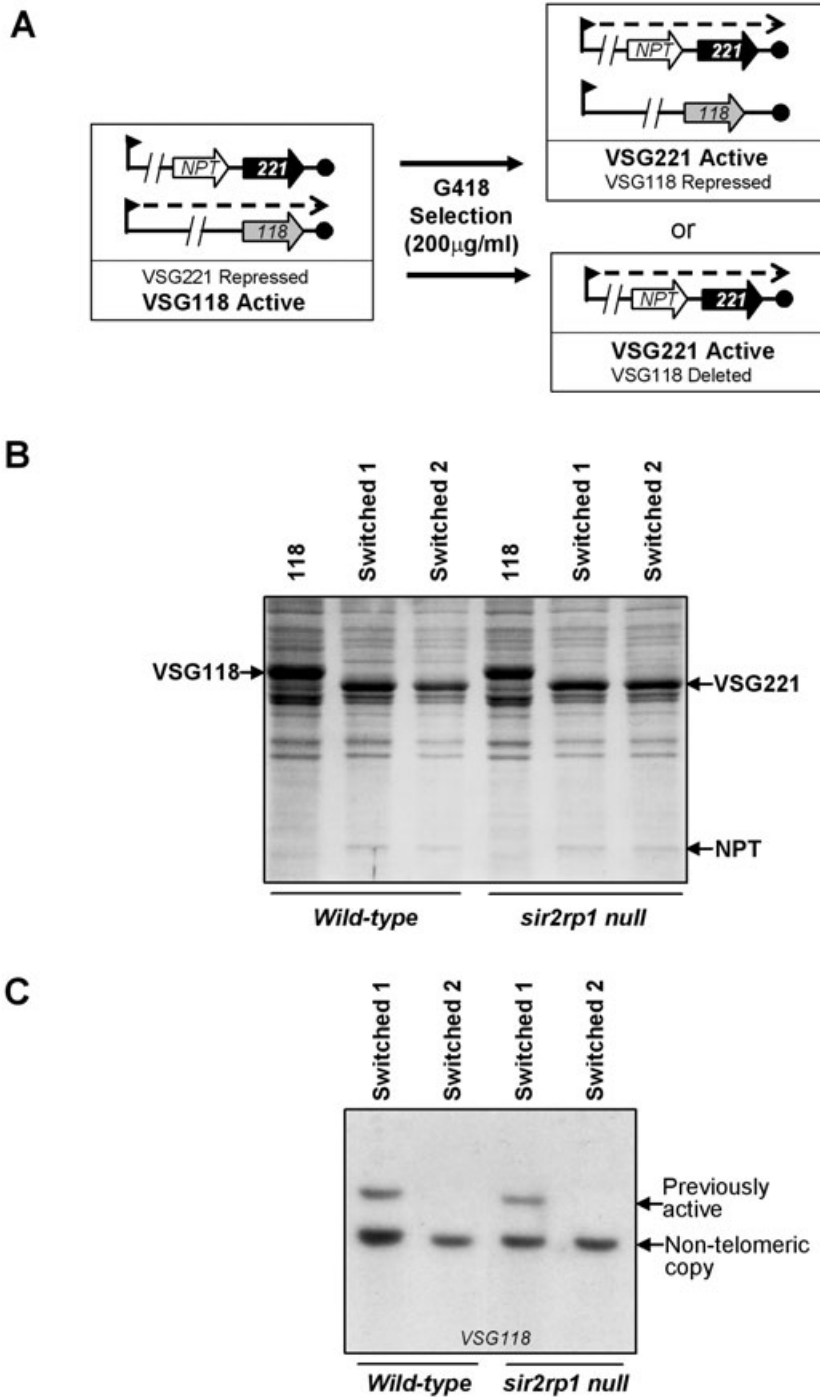
be retained in the presence or absence of SIR2rp1 (5/6 and 2/4 switches derived from wild type and *sir2rp1* null mutants respectively). Examples of *VSG118* gene retention and loss, representing both mechanisms illustrated in Fig. 7A, are shown in Fig. 7C. Retention of *VSG118* indicates that *sir2rp1* null mutants can establish and maintain *VSG118* repression concomitant with *VSG221* activation.

## Discussion

Sirtuins have been linked to a diverse array of biological functions. In human cells, three sirtuins localize to the nucleus, three to the mitochondria and one is microtubule-associated (see Michishita *et al.*, 2005). The picture is not so complex in *T. brucei* and our characterization of all three *T. brucei* SIR2rp's indicates a single nuclear protein and two mitochondrial proteins. It will be important to identify the *in vivo* substrates to further characterize the SIR2rp's. SIR2rp1 clusters in a phylogenetic group with the histone deacetylase, Sir2. Although the p53 deacetylase, *HsSIRT1*, and the tubulin deacetylase, *HsSIRT2* are within the same group, our results are more consistent with histone modification by SIR2rp1 and with the restriction of SIR2rp2 and SIR2rp3 substrates to the mitochondrion.

Sir2 overexpression in *S. cerevisiae* is toxic (Holmes *et al.*, 1997) and we show that SIR2rp1 overexpression in *T. brucei* is toxic. This may be due to inappropriate deacetylation of cytosolic substrates or hypoacetylation of nuclear substrates. Accordingly, appearance in the *T. brucei* cytosol may be due to saturation or loss, via deacetylation, of nuclear-binding platforms. SIR2rp1 overexpression was not shown to be toxic in insect stage *T. brucei* (Garcia-Salcedo *et al.*, 2003) but this could be for either technical reasons or due to stage-regulated function. SIR2rp2 and SIR2rp3 are both mitochondrial proteins and because trypanosomatids are the most ancient branch of the eukaryal lineage to contain conventional mitochondria, our results indicate evolutionary conservation of sirtuins in this organelle. Mitochondrial import may rely upon Arg residues (R<sup>4,16</sup> in *TbSIR2RP2*, and R<sup>2,3,6</sup> in *TbSIR2RP3*) close to the N-terminus (see Hausler *et al.*, 1997). It is now clear that sirtuins can modulate the NAD<sup>+</sup>:NADH ratio, nicotinamide and *O*-acetyl-ADP-ribose levels and these small molecules can in turn influence sirtuin activity. In some cases, the sirtuins may be considered as metabolic sensors, linking cellular energy status to deacetylation and/or ADP-ribosylation activity. The presence of two sirtuins in the *T. brucei* mitochondrion is consistent with conserved roles in energy homeostasis, as is conserved sirtuin-dependent acetylation of acetyl-CoA synthetase (AceCS) (Hallows *et al.*, 2006; Schwer *et al.*, 2006) and ADP-ribosylation of glutamate dehydrogenase (GDH) (Haigis *et al.*, 2006). AceCS and





**Fig. 7.** SIR2rp1 is not required for antigenic variation.

A. The schematic illustrates the expected VSG-switching mechanisms underlying increased G418 resistance/VSG switching. B. Protein extracts from parental (118) cells and two 'switched' clones for each cell type were separated by SDS-PAGE and stained with Coomassie. VSG118 migrates at ~60 kDa while VSG221 migrates at ~55 kDa. Arrows indicate the abundant VSGs and the selectable reporter (NPT) which is also abundant when activated. C. Southern blotting with a *VSG118* probe indicates that both *SIR2rp1* wild-type cells and *sir2rp1* null mutants could inactivate *VSG118* with retention or loss of *VSG118* as illustrated in A.

GDH are mitochondrial enzymes activated by deacetylation and inhibited by ADP-ribosylation, respectively, and similar pathways mediated by SIR2rp2 and/or SIR2rp3 may operate in trypanosomatids.

Primary ADP-ribosylation activity and a role in DNA repair was reported for SIR2rp1 in insect stage *T. brucei* cells in which SIR2rp1 was expressed at approximately double or one third the wild-type level (Garcia-Salcedo

*et al.*, 2003). Using MMS, an alkylating agent that generates DNA lesions, we confirmed a role for SIR2rp1 in DNA repair in insect stage cells and demonstrated a similar role in mammal-infective bloodstream form cells. A role for sirtuins in DNA repair may be conserved from trypanosomatids to mammals since mice lacking Sirt6, another sirtuin with primary ADP-ribosylation activity (Liszt *et al.*, 2005), also display MMS hypersensitivity (Mostoslavsky

*et al.*, 2006). As homologous recombination is important for antigenic variation it will be interesting to determine whether SIR2rp1 can impact on VSG-recombination-related repair mechanisms.

*Saccharomyces cerevisiae* Sir2 is required for telomeric gene silencing (Rusche *et al.*, 2003) and even an archael Sir2 orthologue appears to repress transcription by deacetylating the chromatin protein, Alba (Bell *et al.*, 2002). Sir2 orthologues have also been shown to repress expression of native subtelomeric genes as demonstrated by phenotypic switching in *Candida albicans* (Perez-Martin *et al.*, 1999) and antigenic variation in *P. falciparum* (Figueiredo and Scherf, 2005). In the mammalian host bloodstream, *T. brucei* undergoes antigenic variation. This requires mutually exclusive Pol I-dependent expression of a single VSG gene and the silencing of hundreds of subtelomeric VSG genes, about 20 of which are located downstream of intact promoters. We previously reported 'basal' telomeric Pol I silencing in both major life-cycle stages of *T. brucei* whereas the form of repression that may control antigenic variation is bloodstream form specific (Glover and Horn, 2006). Here, we have shown that SIR2rp1, the only nuclear SIR2rp in *T. brucei*, controls basal telomeric silencing but appears to have little impact on VSG expression site associated repression. *sir2rp1* null mutants maintain repression of a telomere-adjacent VSG, they escape an immune response in mice and they undergo antigenic variation. Thus, the current results indicate two mechanistically distinct forms of repression: SIR2rp1-dependent telomeric silencing and SIR2rp1-independent VSG silencing. We cannot rule out a role for SIR2rp1 in tight VSG repression in natural infections due to the telomere-proximal location of many VSGs and the contribution of SIR2rp1 to telomeric silencing demonstrated here. In addition, a set of VSG genes are transcribed from promoters ~5 kb from the telomere during the first week of a mammalian infection (see Horn and Barry, 2005). It is possible that these 'metacyclic' VSG expression sites are repressed in a SIR2rp1-dependent manner in the insect-stage. Other factors, such as class I/II type deacetylases (Ingram and Horn, 2002) or other histone modifying enzymes and remodelling factors (see table S4 in Ivens *et al.*, 2005) may have a more significant impact on VSG gene expression and silencing in bloodstream-form cells.

As is the case for *TbSIR2rp1*, most sirtuins are dispensable. For example, yeast strains lacking Sir2 and all four Sir2 paralogues are viable (Smith *et al.*, 2000). We were able to generate *T. brucei sir2rp1* null mutants which displayed neither growth nor differentiation defects. *Leishmania* SIR2rp1 however, has been reported to be essential for growth (Vergnes *et al.*, 2005) and was also reported to be found in cytoplasmic granules and to be excreted (Zemzoumi *et al.*, 1998). Although the *T. brucei*

and *Leishmania* proteins are conserved throughout and appear to be homologues (59% identity and 75% similarity), these features contrast with the dispensability and conserved role in DNA repair and transcriptional silencing of *TbSIR2rp1* reported here. Further studies may help to determine whether the two proteins really have such apparently distinct functions.

Histone deacetylases and chromatin modifiers have potentially major roles in controlling gene expression in important human and animal pathogens such as the trypanosomatids. Although some pathogens have co-opted sirtuins to repress subtelomeric virulence factor genes, *T. brucei* has adopted a different, or at least modified, strategy to control the VSG repertoire. However, sirtuin-dependent telomeric silencing in *T. brucei* does suggest widespread conservation of this particular phenomenon. Similar to Sir2 in budding yeast, *TbSIR2rp1* not only represses transcription immediately adjacent to telomeres but also, presumably through relocalization to sites of DNA damage (Martin *et al.*, 1999), controls DNA repair more broadly across the genome. Although SIR2rp1 substrates have not been defined, it is tempting to speculate that this sirtuin represses transcription at telomeres and promotes repair via chromatin modification.

## Experimental procedures

### Trypanosoma brucei growth and manipulation

All cells were derived from Lister 427 bloodstream forms MITat1.2 (clone 221a) and MITat1.5 (clone 118a), grown in HMI-11, transformed with linear DNA constructs and differentiated to the insect stage as previously described (see Alford *et al.*, 2005). For expression of tagged proteins, recombinant vectors were integrated at *RRNA* loci in *T. brucei* cells expressing T7 RNA polymerase (T7RNAP) and/or tetracycline-repressor (TetR) (Wirtz *et al.*, 1999; Alford *et al.*, 2005). Cells expressing T7RNAP were not used for reporter assays (Figs 6 and 7) because this polymerase can compromise reporter cassette silencing (data not shown). Drugs were added ~6 h post transfection at the following concentrations: blasticidin (Invitrogen), 10 µg ml<sup>-1</sup>; puromycin (Calbiochem), 2 µg ml<sup>-1</sup>; G418 (MBI Fermentas), 2 µg ml<sup>-1</sup>; phleomycin (CayLa), 2 µg ml<sup>-1</sup>; hygromycin (Sigma), 2.5 µg ml<sup>-1</sup>. Cell counts were carried out using a haemocytometer. Tetracycline and MMS were from Sigma.

### SIR2RP cloning

All three *SIR2rp* genes used in this study were derived from the Lister 427 *T. brucei* strain. We isolated the *SIR2rp1* gene first using degenerate oligonucleotides; SIR/PF (5'-GGIRTICIGAYTTYMG-3') and SIR20 (5'-TCIARIYYR TCDATRTTYTG-3') (D: A/T/G, I: inosine, M: A/C, R: A/G, Y: C/T); that recognize sequence encoding conserved motifs in SIR2rp's (GI/VPDFR and QNIDG/T/NLE respectively; see

Fig. 1). Temperature was cycled 30 times through 94, 50 and 72°C for 30 s each, in the presence of 100 ng *T. brucei* genomic DNA and *Taq* DNA polymerase (MBI Fermentas). A ~250 bp product was purified and cloned into a pGem-T vector (Promega). Clones that hybridized with the PCR product, representing both alleles, were isolated from a *T. brucei* genomic library consisting of *KpnI* fragments in pBluescript (Stratagene). *SIR2rp2* and *SIR2rp3* (along with *SIR2rp1*) were subsequently identified in the *T. brucei* genome sequence (see table S4 in Ivens *et al.*, 2005).

#### Plasmid constructs

Genes or gene fragments were amplified by PCR from genomic DNA using Vent high fidelity DNA polymerase (New England Biolabs) in conjunction with specific primer pairs (relevant restriction enzyme sites are underlined and start and stop codons are indicated in lower-case) unless stated otherwise. For epitope-tagging constructs, *SIR2rp* tagging primers were as follows:

- i) N-terminal tagging
- *SIR2rp1*: GCTCTAGAACAGAACCGAAGTTAGCAAC and CGGGATCCttACCCTCAACGACTTTTTTC
  - *SIR2rp2*: GCTCTAGAGCTGACCGCCTTGCTATTT and CGGGATCCttCTTCGTGTGCGCCCAAC
  - *SIR2rp3*: GCTCTAGAAGGCGGCCCAATCGTATG and CGGGATCCttTGCCGCGGGACCCTT
- ii) C-terminal tagging
- *SIR2rp2*: GATCAAGCTatgGCTGACCGCCTTGCTAT and GATCTCTAGACTTCGTGTGCGCCCAACTCGA
  - *SIR2rp3*: GATCAAGCTatgAGGCGGCCCAATCGTAT and GATCTCTAGATGCCGCGGGACCCTTTCCGT

The open reading frames (ORFs) were then cloned into the tetracycline-inducible N- or C-terminal tagging (cMyc or eGFP) vectors of the pT7, pT7a and pRPa series (Alford *et al.*, 2005). To generate the *SIR2rp1* gene disruption constructs, we used the *SIR2rp1* genomic clone (-915 nt to +631 nt relative to the ORF). An 873 bp *Sall/Spel* fragment was replaced using *NPT*, *BLE*, *HYG* or *BLA* selectable markers. For expression in yeast, we used the following primers: *SIR2rp1*, GGAATTCATatgACAGAACCGAAGTTAGC and CGGGATCCttACCCTCAACGACTTTTTTC *SIR2rp2*, CCTCGAGTGCAGTTGAGTGATatg and CGAGCTCGGATCCTTCATGTCACTTGGCAC *SIR2rp3*, CCTC GAGGACTGTTTGTAGGTTCAAC and CGGATCCAGC CACTCAAAGTCAAC. The ORFs were cloned in a galactose-inducible expression vector, pTCG, a high-copy vector (2 $\mu$  origin of replication) with a Trp selectable marker. pRn5 was used to integrate a reporter cassette at the *VSG221* locus. This construct was derived from pbRn5 (Horn and Cross, 1997b) by removing the *BLE* gene.

#### RNA and DNA analysis

DNA sequencing was performed using a Thermo Sequenase dye terminator kit (Applied Biosystems), a thermal cycler and an ABI Prism 377 automated sequencer according to the manufacturer instructions. PCR, RT-PCR, Southern and northern analysis were all carried out according to standard

protocols. Signals on Northern blots were quantified using a Phosphorimager (Amersham).

#### Protein analysis

For Western blotting whole cell lysates were separated by SDS-PAGE and electroblotted using standard protocols. Western blots were developed using mouse monoclonal anti-cMyc (4A6, Upstate or 9E10, Santa Cruz) or rabbit polyclonal anti-GFP (Molecular Probes) according to the manufacturers' instructions. Signals were detected using an ECL + kit (Amersham).

For fluorescence microscopy, cells were fixed in 2% (v/v) paraformaldehyde in phosphate buffered saline (PBS) at 4°C for at least 1 h, dried onto glass slides following washing in ice cold PBS and 1% (w/v) BSA in water, permeabilized in 0.5% (v/v) Triton-X100 in PBS for 20 min and blocked in 50% (v/v) foetal bovine serum in PBS for 10 min. Indirect detection of cMyc and eGFP-tagged proteins was carried out using mouse monoclonal anti-cMyc (9E10, Santa Cruz) and polyclonal rabbit anti-GFP (Molecular Probes). Antibody incubations were carried out according to manufacturers' instructions. Cells were mounted in Vectashield (Vector Laboratories) containing the DNA counterstain, 4',6-diamidino-2-phenylindole (DAPI). Slides were analysed on a Nikon Eclipse E600 epifluorescence microscope. Differential interference contrast (DIC) and fluorescence images were captured using a Coolsnap FX (Photometrics) CCD camera and processed in Metamorph 5.0 (Universal Imaging) and Photoshop Elements 2.0 (Adobe). To visualize the mitochondrion, cells were incubated with 100 nM MitoTracker Red CMXRos (Molecular Probes) in HMI-11 at 37°C for 5 min prior to fixation.

#### Yeast assays

*Saccharomyces cerevisiae* strains UCC2 and UCC3505 were grown at 30°C and transformed using the lithium acetate procedure according to standard protocols (see Singer and Gottschling, 1994). For *SIR2rp* induction, glucose was replaced with 3% galactose and cells were pregrown for 4 days at 30°C. For the *ADE2* assay, adenine hemisulphate was at 20  $\mu\text{g ml}^{-1}$  and colonies were incubated for 3 weeks at 4°C for full colour development. For the *URA3* assay, 10-fold serial dilutions in H<sub>2</sub>O were spotted on plates and grown for 4 days.

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