Expression Analysis of the Theileria parva Subtelomere-Encoded Variable Secreted Protein Gene Family

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Abstract

Background: The intracellular protozoan parasite Theileria parva transforms bovine lymphocytes inducing uncontrolled proliferation. Proteins released from the parasite are assumed to contribute to phenotypic changes of the host cell and parasite persistence. With 85 members, genes encoding subtelomeric variable secreted proteins (SVSPs) form the largest gene family in T. parva. The majority of SVSPs contain predicted signal peptides, suggesting secretion into the host cell cytoplasm.

Methodology/Principal Findings: We analysed SVSP expression in T. parva-transformed cell lines established in vitro by infection of T or B lymphocytes with cloned T. parva parasites. Microarray and quantitative real-time PCR analysis revealed mRNA expression for a wide range of SVSP genes. The pattern of mRNA expression was largely defined by the parasite genotype and not by host background or cell type, and found to be relatively stable in vitro over a period of two months. Interestingly, immunofluorescence analysis carried out on cell lines established from a cloned parasite showed that expression of a single SVSP encoded by TP03_0882 is limited to only a small percentage of parasites. Epitope-tagged TP03_0882 expressed in mammalian cells was found to translocate into the nucleus, a process that could be attributed to two different nuclear localisation signals.

Conclusions: Our analysis reveals a complex pattern of Theileria SVSP mRNA expression, which depends on the parasite genotype. Whereas in cell lines established from a cloned parasite transcripts can be found corresponding to a wide range of SVSP genes, only a minority of parasites appear to express a particular SVSP protein. The fact that a number of SVSPs contain functional nuclear localisation signals suggests that proteins released from the parasite could contribute to phenotypic changes of the host cell. This initial characterisation will facilitate future studies on the regulation of SVSP gene expression and the potential biological role of these enigmatic proteins.

Introduction

The subtelomeres of many pathogenic microorganisms contain gene families involved in host-pathogen interactions such as adherence, invasion or escape from immunity (reviewed in [1]). Well-documented examples include the var genes in Plasmodium falciparum [2], the vsg genes in Trypanosoma brucei [3] and the EPA genes in the pathogenic fungus Candida glabrata [4]. The location in telomere-associated regions allows special mechanisms to regulate gene expression. In addition telomere clustering at the nuclear periphery can promote ectopic recombination in telomere-proximal gene families leading to antigenic variation [1].
Heterochromatin, the condensed and transcriptionally inactive form of chromatin, is constitutively present at telomeres and the resulting transcriptional silencing has been described as “telomere position effect” [5]. Post-translational modifications of histones can regulate the extent of condensation and heterochromatin-mediated regulation is one mechanism used by many pathogens to control differential expression of members of subtelomeric gene families [6]. In *P. falciparum*, for instance, the exclusive and alternating expression of one single *iab* gene at a time is under heterochromatin-mediated control [2].

The protozoan parasites *Theileria parva* and *Theileria annulata*, like *Plasmodium* spp., belong to the phylum *Apicomplexa*. Both *Theileria* parasites are transmitted by *isospora* ticks, causing fatal cattle diseases in large parts of Africa and Asia, respectively. After invasion of bovine lymphocytes, *Theileria* sporozoites rapidly eliminate the enclosing host cell membrane and over the next two to three days, the parasite-now free in the cytoplasm-differentiates into a multinucleated schizont [7]. *T. parva* and *T. annulata* schizonts have the unique ability among eukaryotic microorganisms to convert the host cells they infect into a transformed state. This is accompanied by uncontrolled proliferation and resistance to apoptosis. *Theileria*-transformed cells also acquire the capacity to metastasise [reviewed in [8,9]].

While the host signalling pathways mediating these phenotypic alterations have been studied in considerable detail, the parasite factors involved are largely unknown [reviewed in [10,11,12,13]]. In the cytoplasm, the parasite is perfectly positioned to interfere with host cell pathways that regulate proliferation and survival. This can involve events that occur at the parasite surface, as shown for the NF-kB activation pathway [14], or potentially also through secreted parasite proteins [15,16,17].

Secreted proteins are of particular interest as they theoretically have the highest potential for processing by the proteasome and presentation of peptides in association with bovine host MHC Class I molecules. The elimination of *T. parva*-infected lymphocytes by MHC class I-restricted cytotoxic T cell responses is a proven effector mechanism for protective immunity in cattle [18,19]. Therefore such proteins could also function as vaccine antigens and there is preliminary evidence to support this [20,21].

While screening a *T. parva* library using the yeast-2-hybrid system, we made the chance discovery of several members of a novel gene family encoding glutamine (Q)- and proline (P)-rich proteins, most of which contained putative signal peptides for secretion. These proteins were distinct from PIM (polymorphic immunodominant molecule), also called QP-protein [22,23]. Upon completion of the *T. parva* and *T. annulata* genome sequences [24,25], it became clear that the identified genes were part of a large and unique family located in a telomere-associated region of all four *Theileria* chromosomes. The gene family was originally designated subtelomeric variable secreted protein (*SVSP*; gene family. We suggest to designate the family ‘subtelomere-encoded variable secreted proteins’ (*SVSP*) because the genes (rather than the proteins) are clearly subtelomeric and there is no evidence that the (*SVSP*) proteins themselves localise to subtelomeric regions. With 85 members in *T. parva* and 48 members in *T. annulata*, it is the largest family observed in these organisms. Between 2 and 26 genes within this family are located at each of the eight chromosome ends, arranged in tandem arrays with very short intergenic regions of about 200 to 300 base pairs and aligned in centromeric-telomeric direction [24,25]. The organisation of the *SVSP* genes is schematically presented in Fig. S1. An analysis using SignalP software [26] predicts that many *SVSP* have a signal peptide for access to the parasite secretory pathway (Table S2) [25].

The amino acid sequence of a *SVSP*(TP08_0882) containing the characteristic features of this family is presented in Figure 1A. The general structure of *SVSP* consists of a short conserved N-terminal region, in most cases containing a putative signal peptide for secretion, followed by a QP-rich region which is predicted to be highly unstructured (Fig. 1B) [27]. The conserved C-terminus has no significant identity to known proteins and nearly all *SVSP* molecules contain *Theileria*-specific, highly divergent domains termed ‘frequently associated in telomere’ (FAINT). The function of these domains that contain approximately 70 amino acid residues is currently unknown [25].

Information obtained from analysis of the *T. parva* transcriptome by massively parallel signature sequencing (MPSS) technology combined with the fact that we isolated several *SVSP* clones from a cDNA library indicates that many *SVSP* genes are transcribed in the schizont stage [23,28] and data not shown). The analysis by MPSS of *SVSP* transcripts in a cell line transformed by *T. parva* (Muguga) [29] suggested a mosaic-like expression pattern (see Fig. S1 for an overview of *SVSP* gene organisation and expression as reported by Bishop et al. [28]).

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**Figure 1. Schematic presentation of TP08_0882, a typical SVSP.** A. Schematic view and amino acid sequence of TP03_0882. TP03_0882 is 697 amino acids long. The polypeptide has a putative signal peptide (SP) for secretion (purple) with a cleavage site after residue 21 (predicted by the SignalP3.0 web server). A large N-terminal region containing abundant Q and P residues (red) is followed by C-terminal region containing two nuclear localisation signals (NLS 1 and 2, blue) and two FAINT domains from aa 422 to 481 and aa 520 to 579 (green). B. Analysis of the TP03_0882 protein using the FoldIndex® software. The N-terminal region of the protein is intrinsically unfolded, while the conserve C-terminal region is predicted to fold.

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Allocating transcripts from a single infected cell line to individual genes, those expressed at low levels (4-99 transcripts per million) were found to alternate with genes expressed at medium levels (100-999 transcripts per million), and transcribed genes were interspersed with SVSP genes for which no transcripts were detectable.

The present paper provides an initial characterisation of this unusual T. parva gene family. We analysed the expression of SVSP genes in a set of phenotypically different cell lines, established by infection with cloned T. parva parasites, to investigate whether the pattern of expression is conserved or changes depending on parasite genotype, or host cell type and background. Using anti-SVSP antibodies, we present the first evidence for SVSP protein expression. By transfecting mammalian cells with different SVSP constructs, we show that the nuclear localisation signals (NLS) found in several SVSPs are functional and can target SVSPs to different nuclear compartments.

Results
Expression patterns of SVSP genes in different T. parva-infected cloned cell lines

In order to investigate whether SVSP genes are differentially expressed depending on the host cell type the parasite resides in, we compared transcript levels in a CD4+ T cell line (211T-A3) and a B cell line (211B-A3) originating from the animal 211 that was infected with the T. parva (Marikebuni) clone A3 [30]. A microarray was used that contained 70-mer probes corresponding to 78 of the 85 SVSP genes. Seven SVSP genes were not represented on the microarray because appropriate probes could not be detected due to the presence of low complexity regions, lack of uniqueness and/or unsuitable GC content falling outside the required range (Kang’a et al., in preparation). Of the 78 SVSPs analyzed, transcripts of 56 genes were detected (Table S1 and Fig. S1). At the different subtelomeres, the 22 genes with no detectable transcripts were interspersed between transcribed SVSPs. For a block of 9 genes on the reverse strand of chromosome 3, evidence for transcription was absent. However, this region spanning loci TP03_0871 to TP03_0880 also includes 4 genes that were not represented on the microarrays. Two of these 4 genes share 100% identity with another gene (TP03_0871 is identical to TP03_0884; TP03_0879 is identical to TP03_0874) and TP03_0873 is identical to TP03_0878 except for the first 27 bp. As no transcripts were detected for TP03_0874 and TP03_0878 and as probes were designed corresponding to 3‘ regions of the genes it can be concluded that also TP03_0879 and TP03_0873 are not expressed. Because TP03_0871 is identical to TP03_0874, the transcript detected by microarray analysis suggests that one or both genes are expressed. SVSP genes for which no transcripts could be detected were located at all subtelomeres except the reverse strands of chromosomes 1 and 4. Genes with log2 ratios higher than 0.9 (which corresponds to an approximately two-fold difference) were considered as differentially expressed. When the T- and B-cell lines (211T-A3 and 211B-A3) transformed by T. parva (Marikebuni) A3 were compared, for most of the genes no significant difference in transcript level could be detected. Only two genes, TP02_0954 and TP03_0890, showed slightly elevated transcript levels in the T cell line (log2 ratios of 0.9 and 1.4, respectively; Table S1).

Next, SVSP expression in the two T. parva (Marikebuni) A3-infected cell lines was compared to that for a third cell line, 951T-F44, consisting of CD4+ T cells (derived from animal 951) transformed by the T. parva (Marikebuni) clone F44 [30,31]. Compared to the cells transformed with the A3 clone, transcript levels for the majority of SVSP genes were largely conserved in the cell line 951T-F44. However, there were eight genes for which differential expression above the threshold of a log2 ratio of 0.9 was detected (Table S1, Fig. S1). These initial findings indicated that multiple SVSP genes are transcribed simultaneously in cell lines obtained by transformation of T and B cells with cloned T. parva (Marikebuni) parasites. A small percentage of SVSP genes, however, also appear to be differentially expressed.

In order to consolidate the observations obtained using microarrays, we performed quantitative real-time PCR (qRT-PCR) and monitored the expression of 11 SVSP genes using the same total RNA that had been prepared for the microarray analysis. In addition to the genes TP02_0954 and TP03_0890 we analyzed two genes for which no transcripts were detected in microarray experiments (TP02_0953 and TP04_0018), one gene for which no differential expression was observed in all three cell lines (TP03_0882) and the genes TP01_0004, TP03_0869, TP03_0883, TP03_0887, TP04_0007 and TP04_0921, which, according to microarray analysis, were differentially expressed in the cell line 951T-F44. Transcript levels of these eleven genes were compared in the cell lines 211T-A3 and 211B-A3. For the eleven SVSPs analyzed, no significant difference in transcript levels could be observed when CD4+ T cells or B cells transformed by the T. parva (Marikebuni) A3 clone were compared (Fig. 2A). A schematic overview of the position of these genes at the telomeres of chromosomes 1 to 4 is shown in Fig. S1. The small differences in transcript levels of the genes TP02_0954 and TP03_0890 observed by microarray analysis could not be confirmed. In relation to the cell line 951T-F44, the overall SVSP expression pattern in 211T-A3 and 211B-A3 was highly similar (Fig. 2B). For single SVSP genes, however, transcript levels observed in the cell line 951T-F44 differed clearly from that observed for the other two cell lines (Fig. 2B), with some genes showing higher and other genes lower quantities of steady state mRNA. As the cell line 951T-F44 is derived from a different animal and harbours a different parasite clone, it was not clear whether the altered expression pattern could be attributed to the host background, or parasite genotype. To address this question, relative expression levels of five selected SVSP genes were assessed in seven different T. parva-infected cell lines. These included T. parva (Marikebuni) A3 in B cells of two different animals (211B-A3 and 951B-A3), two different T. parva (Marikebuni) clones in y6 T cells of the same animal (951T-I8 and 951T-F31), and three CD4+ T cell lines, each harbouring one of two different T. parva (Marikebuni) clones or a T. parva (Muguga) clone (951T-F44, 951T-F53 and 951T-S80.9) (Table 2). For the five genes analyzed, no difference in transcript levels (with more than two-fold difference) was observed when T. parva (Marikebuni) A3 parasitizing B cells from two different animals was compared (Fig. 3, first two bars in all five panels). By contrast, pronounced differences were detected in cell lines infected with different parasite clones. For instance, in infected CD4+ T cell lines of animal 951, transcript levels of TP03_0869 and TP04_0921 are much lower in the clone T. parva (Marikebuni) F44 than in T. parva (Marikebuni) F53 (to a maximum of an approximately 64-fold difference), while transcript levels of these two genes are comparable in the T. parva (Marikebuni) clone F53 and the T. parva (Muguga) clone S80.9 (Fig. 3, last two bars). On the other hand, TP03_0887 transcript levels are five times higher in T. parva (Marikebuni) F44 than in T. parva (Marikebuni) F53. T. parva (Marikebuni) F31 in the y6 T cell line of animal 951 has an approximately five-fold higher level of TP03_0883 transcripts than T. parva (Marikebuni) B8 on the same host cell background (Fig. 3).

As TP03_0877 belongs to a small region for which specific probes for microarray analysis were not available, transcript levels

 `<p class="plos-table">Table S1. Table showing the expression levels of SVSP genes in different cell lines. The table includes columns for gene name, cell line, and expression level. The expression level is measured as the log2 ratio of the transcript levels in the cell line to the reference cell line. Some genes show significant differences in expression between cell lines, while others do not.</p>`
To test whether the pattern of SVSP expression is stable, transcript levels of TP03_0049, TP04_0018 and TP04_0921 in the F44, F53 and S80-9 genetic parasite background were monitored weekly by qRT-PCR analysis for a period of 3 weeks in several cell lines. Results indicated that the expression pattern of the SVSPs tested is largely stable and, at least in vitro, does not fluctuate significantly over the time period tested (data not shown).

In conclusion, while many SVSP genes appear to be expressed in different T. parva-transformed cell lines, the transcript levels of single SVSP genes can vary considerably depending on the parasite genotype. However, we found no evidence for a possible influence of the host cell background on differential SVSP mRNA expression between parasite clones with the same genotype.

**Detection of T. parva SVSP protein expression**

To investigate whether SVSP expression can also be demonstrated at the protein level, we generated rat polyclonal antibodies directed against the C-terminal conserved domain of SVSP TP03_0882. Since the recombinant protein used for immunisation contained a V5/His epitope tag for detection and purification, the serum was also expected to contain antibodies against V5/His. To confirm that the antiserum contains antibodies against the SVSP backbone, the epitope tag was removed from the recombinant protein using Aε-TEV protease and the resulting protein fragments analysed by immunoblot. A lower molecular weight cleavage product lacking the V5 epitope was readily detectable confirming that antibodies specifically recognizing recombinant SVSP had been generated (Fig. 4A). This was also confirmed by the fact that antibodies reacted with untagged recombinant TP03_0882 protein or with recombinant TY-tagged TP03_0882 protein, but not with other TY-tagged proteins (Fig. 4B). TP03_0882 SVSP could not be detected in extracts prepared from T. parva (Muguga)-infected TpMD409 CD8+ T cells (Fig. 4B, line 6), most likely because only a minority of the parasitised cells express this protein (see below).

Using the anti-SVSP immune serum, we performed immunofluorescence analysis on BoMac cells expressing recombinant TY-tagged TP03_0882 and also on T. parva-infected bovine leukocytes (TpMD409 CD8+ T cells). In BoMac cells, recombinant TP03_0882 was readily detected, localising predominantly to the nucleus of the transfected cell. Fig. 4C shows one example of a BoMac-transfected cell in which the nucleus is strongly stained, except for the nucleoli. In other cells, the pattern of nuclear labelling could be very complex. The different nuclear localisation patterns will be presented separately below. In TpMD409 cells reactivity with schizonts could clearly be demonstrated (Fig. 4D). Co-staining with antibodies against the schizont surface protein PIM indicated that anti-SVSP antibodies predominantly recognised SVSPs located inside the schizont rather than on its surface. A marked dotted pattern, potentially representing the presence of SVSP in vesicles was observed. Anti-TP03_0882 antibodies did not label all parasites. In cultures of T. parva (Muguga)-infected cells, antibodies recognised schizonts in less than 5% of the cells (see also Fig. 4D). One possibility is that not all parasite express TP03_0882. Alternatively, it is possible that in a large number of parasites the protein is expressed, but only at levels that are beyond detection by the anti-SVSP antibodies.

These results provide the first evidence for SVSP expression at the protein level by the *Theileria* parasite.

**The SVSP TP03_0882 targets nucleoli and other compartments within the nucleus of mammalian cells**

In order to test whether SVSP, which are potentially secreted into the host cell cytoplasm, have a tropism for specific host cell structures or compartments, we expressed TP03_0882 lacking the
predicted N-terminal signal peptide in various mammalian cell lines and monitored localisation by immunofluorescence microscopy. Heterologous expression of a full-length TP03_0882-eGFP fusion protein in U2OS cells revealed that the protein almost exclusively localised to the nucleus (Fig. 5A). Within the nucleus different localisation patterns could be observed. In some cells, TP03_0882-eGFP accumulated mainly in compartments resembling the nucleoli (Fig. 5A, left panels), whereas in other cells the protein was found predominantly in the nucleoplasm while nucleoli were clearly excluded (Fig. 5A, right panels). The same localisation patterns were found upon expression of TP03_0882 containing a C-terminal V5 epitope tag, indicating that the nuclear accumulation is not an artefact caused by the eGFP fusion portion of the polypeptide. In addition, similar patterns were observed when anti-SVSP antibodies were used (not shown).

To confirm the nucleolar localisation counterstaining with anti-nucleolin antibodies. In a number of cells, TP03_0882-V5, in addition to targeting nucleoli, was also found to localise to other nuclear structures (Fig. 5C). While a large proportion of TP03_0882-V5 could be found in the nucleoli (recognizable as dark-staining areas by phase contrast microscopy), other, clearly defined spherical structures of unknown origin were also strongly labelled. Finally, in other cells, TP03_0882-V5 failed to associate or associated only weakly with nucleoli and predominantly the spherical nuclear structures were labelled (Fig 5D). Dual labelling with anti-V5, visualising SVSP, and antibodies that recognise telomeres did not reveal any localisation (not shown). Similar localisation patterns were detected in all transfected cell lines examined, including COS-7, HeLa and T. annulata-infected macrophages (not shown). When TP03_0882 constructs lacking the N-terminal 368 aa were expressed, nuclear localisation still occurred, indicating that the QP-rich variable region does not contribute to nuclear translocation (data not shown).
Figure 4. Detection of recombinant and parasite SVSP by immunoblot and immunofluorescence analysis. A. The C-terminal region of SVSP TP03_0882 was expressed as a V5/His-epitope tagged protein in bacteria and subjected to immunoblot analysis using antibodies raised in rats against the same polypeptide (anti-SVSP) or anti-V5 antibodies. Open arrowhead indicates the uncleaved protein; closed arrowhead indicates C-terminal TP03_0882 after removal of the V5/His epitope tag by AcTEV protease. B. BoMac cells were transfected with untagged and TY-tagged full length TP03_0882 and analysed by immunoblot, using anti-SVSP or anti-TY antibodies. The position of SVSP and two TY-tagged control proteins SAG1-mic2 and SAG1-mic20 are indicated. A Ponceau-stained filter is added for loading control and also shows the position of the molecular weight markers. line 1: BoMac transfected with pmaxGFP (Amaxa); line 2: BoMac transfected with pmaxCloning-TP03_0882; line 3: BoMac transfected with pmax-TP03_0882TY; line 4: BoMac transfected with pcDNA-SAG1-Ty-MIC2; line 5: BoMac transfected with pCAN-SAG1-Ty-MIC20; line 6: Whole cell lysate of T. parva (Muguga)-infected cells (TpMD409 CD8+ T cell). C. Detection of TP03_0882 SVSP expressed as a TY-tagged protein in BoMac cells. The nuclei of two cells are stained by DAPI. The cell on the left expresses SVSP as indicated by its reactivity with TY antibodies. The cell is also labelled after staining with anti-SVSP antibodies, showing nuclear localisation of the expressed protein. Scale bar: 20 μm. D. Cytospin preparations of T. parva (Muguga)-transformed cells (TpMD409 CD8+ T cells) mixed with uninfected bovine control BL20 cells were fixed in paraformaldehyde and analyzed by indirect immunofluorescence using anti-SVSP antibodies (green) and anti-PIM antibodies (red). Host and parasite nuclei were stained with DAPI (blue). The control cell in the middle does not harbour a parasite and is negative for PIM and SVSP. Both the cells at the top and the bottom are parasitised and thus labelled with anti-PIM. Only the parasite in the cell at the bottom, reacts with antibodies raised against TP03_0882 SVSP (green) partly revealing a vesicular staining pattern. Merge is an overlay of the three panels. Scale bar: 10 μm doi:10.1371/journal.pone.0004839.g004
Functional analysis of potential, parasite-encoded nuclear localization signals

Importation of proteins into the nucleus is classically mediated by recognition of nuclear localisation signals (NLS), which fall into two major classes. Analysis of the TP03_0882 amino acid sequence using the PsortII [34] and NucPred [35] prediction programs revealed the presence of two potential NLS. The NucPred algorithm predicted an SV40 large tumor antigen-type NLS (designated NLS1, aa 340–351; see Fig. 1). A bipartite NLS (aa 394–410; NLS2) was detected using the PSORTII algorithm. The presence of NLS is not specific for this individual gene, since using both algorithms, putative nuclear import motifs could be found in 78% of the T. parva SVSP family members (Table S2).

To investigate to what extent these NLS contribute to targeting TP03_0882 to the nucleus, constructs lacking either NLS1 (pTP03_0882ΔNLS1), NLS2 (pTP03_0882ΔNLS2) or both (pTP03_0882ΔNLS1+2) were generated and the sub-cellular localisation of the V5-epitope tagged proteins monitored by

Figure 5. SVSP TP03_0882 expressed in U2OS cells localises to different nuclear compartments. A. TP03_0882 was expressed as eGFP fusion protein in U2OS cells. Cells growing on coverslips were fixed with paraformaldehyde. DNA was stained with DAPI (blue). Of the two cells shown in the panels on the left, one expresses SVSP which is localised to the nucleoli. The panels on the right show a cell with a nucleoplasmic SVSP localisation pattern. Scale bar: 20 μM. B. Cells transfected with a plasmid encoding V5-tagged SVSP TP03_0882 were fixed with methanol and stained with antibodies directed against nucleolin (as nucleolar marker, green) or V5 (SVSP-expressing cell, red). The overlay (merge) reveals the colocalisation of SVSP and nucleolin in the nucleoli. Scale bar: 25 μM. C. In some transfected cells, SVSP (green) localises to nucleoli (white arrows) and other nuclear structures (orange arrows). In the left panel, nucleoli were visualized by phase contrast (dark areas). In the middle panel, SVSP is visualised using anti-V5 antibodies. Merge indicates the overlay of both panels. Scale bar: 20 μM. D. In some cells, SVSP (anti-V5, green) does not localise to the nucleoli (stained with anti-nucleolin, red) and is only found in dense nuclear bodies. Nuclei are stained blue (DAPI). Scale bar: 20 μM.

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immunofluorescence microscopy in transfected U2OS cells. Upon deletion of NLS1, the protein still translocated to the nucleus, but, in contrast to wild-type TP03_0882-V5, a large proportion of the protein was retained in the cytoplasm (Fig. 6). Similar observations were made when NLS2 was deleted (pTP03_0882ΔNLS2). Upon removal of both nuclear localisation motifs (pTP03_0882ΔNLS1ΔNLS2), however, the protein was to a large extent excluded from the nucleus, indicating that NLS1 and NLS2 can function independently, but that both motifs are required for optimal and efficient import of TP03_0882 into the nucleus. For a minority of other members of the SVSP family such as TP02_0955, PSORTII or NucPred failed to identify aa sequences predicting an NLS. When epitope-tagged TP02_0955-V5 was expressed in U2OS cells, the protein remained almost exclusively in the cytoplasm, similar to TP03_0882ΔNLS1ΔNLS2.

Discussion

This study presents an initial characterisation of the expression of the T. parva SVSP family, the largest gene family identified in Theileria so far [24,25]. We found that in three different cell lines infected with cloned T. parva (Marikebuni) parasites, transcripts corresponding to more than 65% of SVSP genes are expressed simultaneously. This is in agreement with the findings of a whole-transcriptome analysis by MPSS, performed on a T. parva (Muguga)-infected cell line [28] which showed that SVSP mRNA can be detected at low to medium levels and also that genes with similar expression levels are distributed over the eight subtelomeres showing no clustering at specific locations.

While SVSP transcript levels in different infected cell lines appear to be largely comparable, specific SVSP genes exhibiting differential expression were identified. We investigated the effects of three variables that could conceivably have an impact on SVSP expression levels: the individual animal background the infected lymphocytes were derived from, the host cell type (T or B lymphocyte) and the parasite genotype. The latter was found to be the key determinant of differential expression between copies within the gene family. Cell lines containing clonal parasite genotypes showed SVSP mRNA expression patterns that differed between the clones, but were not affected by host cell type or animal background (Figs. S1 and 2). The most pronounced differences were found for the SVSP genes TP03_0869 and TP04_0921, located on the reverse strands of chromosomes 3 and 4. When compared to other SVSPs, the only obvious difference is that in TP04_0921, the QP-rich region is lacking, which is not the case for TP03_0869. The absence of the QP-rich region has also been observed for a few other SVSPs, however, and is unlikely to explain the differences in mRNA expression. Like most SVSPs, TP03_0869 and TP04_0921 contain a predicted signal peptide potentially allowing access to the secretory pathway, two FAINT domains in the C-terminal region and no predicted transmembrane domains, which is consistent with secretion. While TP03_0869 has no special position in the SVSP array, TP04_0921 is the most telomere-proximal SVSP gene on the reverse strand of chromosome 4. Although no absolute quantification was carried out, qRT-PCR experiments revealed that, compared to all other SVSP genes, the Cq values of TP03_0869 and TP04_0921 are much higher in the cell line 951T_F44, compared to other cell lines. This suggests that transcription of the two genes is markedly downregulated in 951T_F44 rather than upregulated in the other cell lines.

The sequences of TP03_0869, TP03_0883 and TP04_0921 (showing the highest differences in transcript levels between the different parasite genotypes) and the intergenic region between

Figure 6. Identification of two functional NLS in SVSP TP03_0882. U2OS cells were transfected with plasmid constructs encoding V5-tagged forms of TP03_0882 containing both NLS (TP03_0882), lacking NLS1 (TP03_0882ΔNLS1), lacking NLS2 (TP03_0882ΔNLS2) or both NLS (TP03_0882ΔNLS1ΔNLS2). In addition, cells were also transfected with a plasmid encoding TP02_0995, an SVSP predicted not to contain a NLS. Cells were fixed in methanol and SVSP localisation monitored using anti-V5 antibodies (green). DNA was stained with DAPI (blue). doi:10.1371/journal.pone.0004839.g006
Theileria SVSP Expression

TP04_0928 and TP04_0921 are conserved between the different parasite genotypes, indicating that differences in mRNA expression are not caused by polymorphism. Polymorphism is not unexpected as it is prevalent in clusters of neighbouring genes of the 3′S fragment related family which are also subtelomeric [33]. We also established that mRNA expression patterns appear to be fairly stable as mRNA expression of three SVSP genes tested in different cell lines remained largely constant over an observation period of up to 8 weeks. Whether SVSP expression in cloned parasites would also be constant in vivo, represents an interesting issue for future investigation.

It will be of interest to determine whether individual parasites express single SVSP mRNAs, or whether a selection of different SVSP genes can be expressed by a single schizont. Also, since schizonts are multicellular, it remains to be established whether individual SVSP genes are transcribed in one or several schizont nuclei within a single infected cell.

Raising antibodies to SVSPs proved very difficult and required multiple attempts in different animal species. This is in marked contrast to the schizont surface protein PIM, which also has a QP-rich domain and –as indicated by its name–is very immunogenic, both in cattle and mice. The observation that polyclonal antibodies directed against the SVSP TP03_0882 show strong staining in only a small number of parasitized cells indicates that not all parasites express the protein or that expression levels vary considerably between individual parasites. It also suggests that antibodies directed against the structurally conserved SVSP C-terminal domain encoded by TP03_0882 probably do not generally cross-react with other SVSPs. At this stage we cannot rule out the possibility that SVSP TP03_0882 is expressed at low levels below the detection threshold of immunofluorescence analysis in additional cells, or that more than one SVSP is expressed per parasite. Similar results were obtained when other cell lines were investigated, suggesting that restricted and/or strongly varying levels of expression is a typical feature of SVSPs.

Immunofluorescence analysis showed a clear accumulation of TP03_0882 SVSP in vesicular structures, supporting the notion that SVSPs are incorporated into the parasite secretory pathway. Transfection studies in mammalian cells also confirm that the predicted NLS could help target SVSPs to the host cell nucleus. Nevertheless, under the conditions used, the anti-SVSP antibodies that we generated did not allow us to confirm that SVSPs are indeed secreted into the host-cell cytoplasm and translocate to the host cell nucleus. It is possible, that only small amounts of SVSP are secreted and that our detection tools are not sensitive enough. Alternatively, it is possible that secreted SVSPs are rapidly degraded resulting in low steady state levels of protein.

SVSPs are a unique family with no known orthologues in genera other than Theileria. The function of both the unstructured N-terminal QP-rich region and the conserved C-terminal region containing the FAINT domains are unknown. The fact that the QP-rich region of the protein is lacking in some SVSPs suggests that this variable region is not indispensable for protein function. It also indirectly supports the notion that the conserved C-terminal domain, which also contains the NLS, could be the main contributor to the still unknown-biological function(s) of SVSPs. It is intriguing to note that TP03_0882 localises to defined nuclear structures when expressed in mammalian cells. Nuclear localisation has also been observed for other Theileria proteins such as TashAT and SuAT proteins [13,16,36,37]. Ectopically expressed TP03_0882 was frequently found to localise to the nuclei. The nucleolus is a multifunctional subnuclear compartment that is best known as the site where ribosome biogenesis takes place. However, different studies also point towards an involvement of nucleoli in processes linked to cell cycle control, senescence, DNA replication and repair, and the regulation of stress responses (reviewed in [38]). The possibility that Theileria proteins might target the host cell nucleolus is interesting in view of the fact that several viral proteins, including the Rex protein of the transforming human T-lymphotropic virus (HTLV-I) [39] and a portion of the adeno-associated virus protein Rep [40] can also be found in nucleoli. TP03_0882 did not localise to nucleoli in all cells, however, and was sometimes found in association with other nuclear structures. Each cell cycle, the nucleolus undergoes extensive reorganisation and the nucleolar proteome is also subject to dynamic changes dependent on cellular conditions [41]. Such changes might represent a factor in determining the pattern of TP03_0882 localisation to the nucleolus. Alternatively, nucleolar targeting of TP03_0882 might be subject to cell cycle-dependent changes in post-translational modifications (see [38] and references therein).

Whether SVSP genes are expressed in a mutually exclusive manner as has been described for var genes of P. falciparum remains to be established. Var genes, many of which are also subtelomeric, encode PEMP1, a surface protein involved in adherence of infected erythrocytes to epithelial cells or to other erythrocytes. Only one PEMP1 is expressed at the time and expression involves multiple layers of control involving chromatin modifications, nuclear positioning, and promoter-intron interactions [2,42,43]. Switching PEMP1 expression allows intra-erythrocytic Plasmodium Plasmodium to avoid clearance by the host spleen. Rather than being expressed on the surface, SVSPs are predicted to be secreted into the host cell cytoplasm. Proteins secreted by the Theileria schizont can access the MHC class I–CD8+ T cell surveillance system which plays a central role in immunity to T. parva. However, despite the theoretical abundance of potential candidates [21], in individual animals, this immunity is tightly focused on only a few immunodominant MHC–peptide epitope combinations (reviewed by [19,44] leading to a limited cross-protection between different strains. At this stage, it is not known how efficiently SVSPs are processed for presentation through the MHC class I pathway. Nevertheless, assuming each individual SVSP released into the host cell cytoplasm could potentially provide epitopes capable of priming protective cytotoxic responses, the simultaneous release of a large number of similar SVSP proteins would by definition increase the chances of detection and elimination of parasitised cells. Our initial findings using antibodies directed against TP03_0882 suggest that this specific SVSP is expressed by only a small fraction of the parasites. Although we cannot exclude the concurrent expression by the same parasite of additional SVSPs, our initial findings indicate that the expression of individual SVSPs may be restricted. It has been shown that aminopeptidases in the endoplasmic reticulum that process antigenic precursors generated by proteasomal degradation, thus generating peptides suitable for presentation by MHC class I molecules, fail to trim prolines that flank the NH2-termini of these precursors [45]. To what extent prolines in the QP-rich region of SVSPs might interfere with efficient peptide generation remains to be established.

In conclusion, using cell lines derived from individual T. parva clones, we provide a first characterisation of the expression pattern of members of this large and intriguing T. parva gene family. SVSP genes are also found in the closely related parasite T. annulata, potentially reflecting a conserved biological role. The tropism SVSPs show for different sub-nuclear compartments of the mammalian cell could provide important clues concerning their biological function.
Materials and Methods

Cell lines and parasites

Cloned *T. parvo*-infected cell lines used for analysis of *SVSP* transcript patterns (Table 2) were generated and cultured as described [29,30,31]. Parasite genotypes A3, F31, F44, F53 are referred to as 72-01, 72-30, 72-27 and 72-29 respectively in [31] and originate from stabitate 72. The cell line 951T_I8 was generated from a different *T. parvo* (Marikebuni) stabitate originally obtained from Kenya Agricultural Research Institute (KARI). The *T. parvo* (Muguga) clone 80.9 was newly generated and is not published. Parasite genotypes were determined with *T. parvo*-specific satellite markers [31,46] and host cells were typed using flow cytometry. Native *SVSP* were detected in *T. parvo* (Muguga)-infected CD8+ T cells [47]. Expression of recombinant *SVSP* was analyzed in U2OS and BoMac cells cultured under standard conditions.

Microarray and quantitative real-time PCR analysis

Total RNA was extracted from schizont-infected cells using the RNeasy Mini Kit according to the manufacturer’s protocol (QIAGEN). Each infected cell line was represented by two biological replicates. RNA was treated on-column with RNase-free DNase (QIAGEN) to remove contaminating genomic DNA. Conventional end-point PCRs were performed with *T. parvo* actin (5’-CGGATCCCGACACAAATGTGCA-3’ and 5’-CGGATTCAGGTGGCAAAATATT-3’) and PIM primers (5’-CGAAGCTTGAAAACATGAAGATCTTTCCC-3’ and 5’-TACGGCGCCGCTTAACAAAAATCTCTTTGGAATGCG-3’) using GoTaq DNA Polymerase (Promega) to detect contamination with genomic DNA. The RNA was considered ‘DNA-free’ when no amplification product was detectable after 30 cycles.

For microarray analysis, a direct two-colour experimental design was used to compare parasite transcripts levels using 70-mer oligonucleotide microarrays containing 4060 *T. parvo* genes (Kang’a et al., in preparation). Hybridisations were performed using 15–20 μg of Cy-dye labelled amplified-RNA (aRNA) from each infected cell line. Briefly, cDNA targets were first prepared from 5 μg of total parasite RNA extracted from each *T. parvo*-infected cell line by reverse transcription followed by in vitro transcription with simultaneous incorporation of amino-allyl dUTP using the Aminoallyl Message Amp RNA amplification kit (Ambion Inc., Austin TX) according to manufacturer’s instructions. The resulting aRNA was fluorescently labelled using Cy3 and Cy5 dyes through coupling with amino-allyl dUTP in 0.1M Sodium Carbonate buffer. The labelled targets were used to hybridise the 70-mer oligonucleotide probes on the glass slides by incubation for 16 h at 42°C in a hybridisation buffer consisting of 50% formamide, 5% SSC, 0.1% SDS, and 0.6 μg ml⁻¹ salmon sperm DNA. Flip-dye hybridisations were performed by reversing Cy-dyes for each pair of hybridised parasite aRNA. Following a 16 h incubation, slides were sequentially washed in 2× SSC, 0.1% SDS for 5 min at 42°C, 0.1× SSC, 0.1% SDS for 5 min at room temperature, 0.1× SSC for 5 min at room temperature and dried by centrifugation. Scanning and data extraction was performed using GenePix® 4000B Axon scanner and Genechip 5.0 software respectively (Axon Instruments, Union City CA).

Data were analyzed using the marray packages from the Bioconductor site (www.bioconductor.org; [48]). Following data normalisation, executed in two steps, first using within-slide loess normalisation followed by quantile between-slide normalisation, we employed linear models [49] to rank the differentially expressed genes in order of evidence (i.e., P value) of differentially expressed genes. Briefly, the function lmFit in limma accepts a model matrix, which describes the experimental design and produces an output object of class MarrayLM, which stores the fitted model information for each gene. The fitted model object is further processed by the eBayes function to produce empirical Bayes test statistics for each gene, including moderated t-statistics, P-values and log-odds of differential expression. Genes with P<0.05 and with log2-fold change (M) value of <-0.9 or >0.9 (representing approximately 2 fold change in transcript abundance) were considered as significantly differentially transcribed. Since each gene on the array was printed in quadruplicate, the final list of unique genes was generated by calculating the medians of in-slide replicates for genes that met these criteria. Accession Nr: GEO14093.

For quantitative real-time (qRT-PCR), first strand cDNA synthesis was performed from 2 μg total RNA using RNase H deficient M-MLV reverse transcriptase and 1 μg oligo(dT) primer in the presence of RNasein Ribonuclease inhibitor as described by the manufacturer (Promega). Reverse transcriptase was heat-inactivated for 5 min at 80°C and to verify the generation of cDNA products, end-point PCR reactions were performed as described above.

Transcript levels of selected *SVSP* were determined by qRT-PCR using SYBR green dye technology (Applied Biosystems). The Primer Express Software v.2.0. (Applied Biosystems) was used to design the primers shown in Table 1. The oligonucleotide sequences were analyzed for specificity using BLAST searches (www.ncbi.nlm.nih.gov/BLAST). Since the housekeeping genes fructose bisphosphate aldolase (FBA, TP01_0101) and actin (TP02_0905) are highly conserved in a wide range of species, coding sequences of the *T. parvo* and bovine homologues were aligned using CLUSTALW at EMBnet-CH and sequences with low similarity were selected manually. Primer pairs for these genes were subsequently designed with the Primer3 software [50]. For the *SVSP* TP04_0007, no sequences were found which do not at the same time occur in at least one other *SVSP* gene. For this gene, primers were designed such that the forward primer does not cross-hybridize with the same genes as the reverse primer. All oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Since the *SVSP* genes contain highly conserved regions, primer pairs were first tested and only primer pairs were used for which single melting peaks were obtained.

| Locus Name | Forward Primer | Reverse Primer |
|------------|----------------|----------------|
| TP01_0004  | tgaatgtgctgagacaaacc | gacctcactttcgcgagtgg |
| TP02_0953  | tgtcttctggctgacgata | tctatgggatatgccgagtta |
| TP02_0954  | aagggctcctggcgcacact | gccgtgggtcgtctgataa |
| TP03_0869  | tgttttaaatatggtaagaaatag | agtcttccttcgagtctcagaa |
| TP03_0877  | cactggagctctttaaatatgtgc | cccactcatacgcagcacactt |
| TP03_0882  | ccatacgactcaccagattcatag | ccttttcctcatcttgcgtaa |
| TP03_0883  | acgtgtctttctggcactgatctc | tctctctctcatctttggtgga |
| TP03_0887  | tccgggtatcaccaggtt | gaaattgtgtaatgctggagcagtta |
| TP03_0890  | gggtcctcaaaatatgtgctt | atgtgtgtaacagacgataac |
| TP04_0007  | tggaacagtgtgagaatgtaatag | tctctcattgtgagtgtaaat |
| TP04_0018  | ccccgaccaattacagtggcaac | tgtagccgaaaaacaggcatcgaga |
| TP04_0921  | cataaataagcttgataaatacgtgctgta | cctgagcttacaccaggccttatac |

Table 1. Primer pairs used for quantitative RT-PCR
The efficiency of amplification was assessed for each primer pair by generating standard curves plotted as C<sub>t</sub> versus log concentration. Only primers and primer concentrations yielding a slope between −3.2 and −3.6 were used.

All qRT-PCR reactions were performed in duplicates in a total volume of 25 μl containing Power SYBR Green Master Mix (Applied Biosystems) and 250 or 500 nM of each primer (depending on reaction efficiency). For quantification of transcript levels in the different cell lines, 40 ng cDNA were used per reaction. The assays were carried out with the ABI Prism 7700 Sequence Detector System and associated Sequence Detection Software v.1.9.1 (Applied Biosystems) following the manufacturer’s guidelines. For each cDNA sample, a reaction was carried out with the corresponding amount of input RNA without reverse transcription and for each reaction a melting temperature profile was generated to ensure the amplification of a single product. To detect unspecific amplification of bovine DNA, control reactions were performed using cDNA of an uninfected bovine cell line (BL20) as template.

For final quantification of the relative transcript levels, the comparative C<sub>t</sub> method was applied using fructose-bisphosphate aldolase (TP01_0101) as an endogenous control and the sample with the lowest expression level of the respective gene as calibrator.

### Generation of anti-SVSP antibodies

The C-terminal part (bp 1075–1821, AA 359–607) was amplified with primers 5′-GACCGATGAAACTACT-GATCACC-3′ (sense) and 5′-TTATCCTATATTGGCTTT-TATTAATT-3′ (antisense) using pTP03_0882 as template. The PCR fragment was directly cloned into pET151/D-TOPO (Invitrogen) a bacterial expression vector resulting in pET151-TP03_0882C. The PCR product was over-expressed in E. coli BL21 star cells (Invitrogen) and the 6xHis tagged protein was purified over Protino Ni 150 columns (Machery Nagel, #745 100.10). The purified protein was mixed with GERBU adjuvant (GERBU Biotechnik GmbH, Gaiberg, Germany) and rats were immunised. The diluted immune serum was used for immunofluorescence and Western blot analysis. These polyclonal antibodies were not necessarily mono-specific for TP03_0882 and may share with other SVSPs. In order to cleave the V5/His–tag from recombinant TP03_0882C-V5 40 μg of purified protein was incubated for 3 h at 30°C in the presence of 20 U of AcTEV protease, 1 mM DTT and 1xTEV buffer (Invitrogen).

### Recombinant SVSP constructs

Different versions of TP03_0882 and TP02_0995 were expressed as recombinant proteins in mammalian cells either tagged with V5 or TY epitopes or as eGFP fusion proteins. All constructs expressed in mammalian cells lack the predicted signal peptide for secretion. To generate pTP03_0882, PCR was performed with the primers 5′-ATAAGCTTATGCGCTGAT-CAACCTGCTGATGAT-3′ (sense) and 5′-GCCGTCGACCTCA-TATTTGGCCTTTATTAATT-3′ (antisense) using genomic DNA from the T-cell line 803 containing T. parva (Muguga) as template. Since in preliminary experiments we had initially isolated an incomplete cDNA clone corresponding to TP03_0884, the DNA sequence of the primers was based on the TP03_0884 gene locus, also a member of the SVSP gene family. As all SVSP family members have very conserved 5′- and 3′-ends in our PCR reaction TP03_0882 was amplified instead, resulting in a protein with the following changes compared to the original TP03_0882 protein sequence published in the TIGR T. parva genome database: S30A, E31D, T607R, all conservative amino acid changes, introduced by using the above described primers. We found two additional changes in our PCR fragment by comparing the DNA sequence to the TP03_0884 sequence in the TIGR database. Both changes are A to G transitions which lead to the following amino acid changes: D45G and T158A. The PCR product was directly cloned into pCR®2.1-TOPO (Invitrogen) resulting in pTOPO-TP03_0882 and from there sub-cloned into the mammalian expression vector pcDNA3.1/V5-HisA (Invitrogen) using the restriction enzymes EcoRI and HindIII. The final construct produces a truncated version of TP03_0882 from amino acid 26 to 607 containing the above-indicated changes and is referred as TP03_0882 in this paper. pTP03_0882 was used as template for constructing three plasmids coding for TP03_0882 versions lacking either one of the two putative NLS or both. To construct pTP03_0882ANLS1 the following primers were used to amplify the N-terminal part (bp 26–1098) of TP03_0882: 5′-TATACGACTCATATAGG-3′ (sense), and 5′-GTGTCGATG-GAGTTGATTTTTTGAATGATTTGTTGTTTGGCTTT-TATTAATT-3′ (antisense) and the two following primers were used to amplify the C-terminal part (bp 1141–1821): 5′-GCTGCAGAGATCAGAAAGACAGAAAGATAATCAGAGAAGATTTG (sense) and 5′-GCCGTCGACCTCA-TATTTGGCCTTTATTAATT-3′ (antisense). The amplicon encoding the N- terminal section was digested with the restriction enzymes EcoRI and NolI, the fragment corresponding to the C-terminal was digested with NolI and XhoI. Both fragments were ligated simultaneously into pcDNA3.1/V5-His linearized with EcoRI and XhoI. The same cloning strategy was used to construct

### Table 2. T. parva-infected cell lines established by transformation with different T. parva clones.

| Cell line | Parasite isolate | Parasite genotype | Animal | Host cell type | Reference |
|-----------|------------------|-------------------|--------|---------------|-----------|
| 211T_A3 (211-72-29) | Markebuni | A3 | 211 | CD4+ | [30] |
| 211B_A3 (211-72-33) | Markebuni | A3 | 211 | B cell | [30] |
| 951T_F44 (951-F44) | Markebuni | F44 | 211 | CD4+ | [31] |
| 951B_A3 (951-A3) | Markebuni | A3 | 951 | B cell | [31] |
| 951T_L8 (951-L8) | Markebuni | L8 | 951 | γδ | [31] |
| 951T_F31 (951-F31) | Markebuni | F31 | 951 | γδ | [31] |
| 951T_F53 (951-F53) | Markebuni | F53 | 951 | CD4+ | [31] |
| 951T_S80.9 (951-S80.9) | Muguga | 80.9 | 951 | CD4+ | none |
| TpMD409 | Muguga | D(409) | 951 | CD8+ | [47] |
pTP03_0882ANLS1+2. The same N-terminal part (bp 26–1098) as used for construction of pTP03_0882ANLS1 cut with EcoRI and XhoI was ligated with the C-terminal part (bp 1231–1821) amplified with the primers 5'-GGATCCCATGGAATATCTGGAAGACCTTG-3' (sense) and 5'-GGCTGCAGCTTATATTGGCCTTATTAAATTT-3' (antisense) and cut with NolI and XhoI into EcoRI/XhoI linearized pCDNA3.1/V5-His. The first pTP03_0882ANLS2 construct was made as GFP fusion protein in the vector pcDNA3-N3 (BD Biosciences Clontech). Amplification of the N-terminal part (bp 26–1179) was performed with the primers 5'-TAATACGACTCACTATAGG-3' (sense) and 5'-GGATCCCATGGAATATCTGGAAGACCTTG-3' (antisense). The C-terminal part (bp 1231–1821) was amplified with the primers 5'-GGCTGCAGCTTATATTGGCCTTATTAAATTT-3' (antisense) and cut with NolI and BamHI, both fragments were ligated simultaneously to EcoRI/BamHI linearised pcDNA3-N3 to generate pTP03_0882ANLS2-EGFP. This vector was used to generate pTP03_0882ANLS2pTP03_0882ANLS2-EGFP was cut with HindIII to cut out a 1176 bp fragment harbouring the ANLS2 truncation. This ANLS2 HindIII fragment was ligated into HindIII cut pTP03_0882 replacing the N-terminal part containing NLS2. The resulting vector is called pTP03_0882ANLS2. For expression of the pTP03_0882 as a GFP fusion protein, the cDNA was subcloned from pTOPO-pTP03_0882 using the restriction enzymes SalI and XhoI and ligated into pEGFP-N3 cut with the same enzymes. The resulting vector is called pTP03_0882-eGFP. We also cloned a gene of the SVSP family, which does not originally contain a NLS, Tp02955 a single exon gene. The gene lacking the N-terminal region coding for the signal peptide for secretion (bp 58–1683) was amplified using the primers 5'-AATTATCTGGGAAAGCTTG-3' (sense) and 5'-TGAC-CTGGAATAATTGTTTTAGCTTCTA-3' using genomic DNA of the T-cell line 951-F31 harbouring T. parva chromosomes. Theileria parva inactivated FCS (FCS/PBS) for 10 min. Anti-PIM 40.2 antibodies (1:1000; Invitrogen, Molecular Probes A-11006) or mouse anti-V5 antibodies (1:500; Invitrogen life technologies R960-25), to visualize nucleoli rabbit anti-C23 (H259) antibodies were used (1:200; Santa Cruz sc-13057). Secondary antibodies were used at the following dilutions: Alexa488-conjugated goat anti-rabbit antibody (1:1500; Molecular Probes A-11034), Alexa488 conjugated goat anti-mouse antibody (1:1500; Molecular Probes A-11029), Texas Red-conjugated goat anti-mouse antibody (1:1500; Invitrogen, Molecular Probes T862). To visualize eGFP fusion proteins cells were fixed in 4% paraformaldehyde for 10 min at room temperature. DNA was stained with Dapi and coverslips were mounted onto slides using mounting reagent (Vectashield). All samples were analyzed using a Nikon Eclipse fluorescence microscope 80i and digital images were generated and processed using the OpenLab 3.1.5 software.

Immunoblot analysis

Whole cell lysates of T. parva-infected TpM409 CD8+ T-cells or transfected BoMac cells were prepared in 1× Lamml-buffer containing 4% β-mercaptoethanol. The lysates were sonicated and insoluble cell debris was removed by centrifugation at 23'000 g for 10 min at 4°C. Samples were stored at −20°C and aliquots of 20 μl cell lysate (2×10^6 cells) were used for Western blot analysis. Immunoblot analysis was performed according to standard procedures. Samples were boiled for 5 min before loading onto a 10% SDS-polyacrylamide gel. After blotting, the nitrocellulose membranes were blocked for 1 h with 5% milk-TBST (10 mM Tris, pH 8.0; 150 mM NaCl; 0.05% Tween-20). The membranes were incubated in the same blocking buffer containing rat anti-SVSP immune serum diluted 1:500, mouse anti-TY antibodies 1:2000 (D. Soldati, Geneva, Switzerland) or mouse anti-V5 antibodies (P/N 46-0705, Invitrogen) 1:2000 over night at 4°C. Membranes were washed 3× for 5 min in TBST and then incubated for 1 h at room temperature in 5% milk-TBST containing secondary antibodies: horseradish peroxidase-conjugated goat anti-rat antibody (SC-2006, Santa Cruz Biotechnology) or horseradish peroxidase-conjugated rabbit anti-mouse antibody (P0260, Dako) both diluted 1:2000. Membranes were washed 3× for 5 min with TBST and once for 5 min with TBS (10 mM Tris, pH 8.0, 150 mM NaCl). For visualisation of the signal in the FUJIFILM LAS-3000 system, the ECL detection system was used according to the manufacturer’s instructions (Amersham Biosciences).

Supporting Information

Figure S1 Schematic presentation (adapted from Bishop et al., 2005) depicting the organisation of SVSP genes at the subtelomeres of the four T. parva chromosomes. SVSP genes are shown in red and the numbers represent the original numbering allocated to each of the genes encoded by the T. parva (Muguga) SVSP genes.
Table S1  Microarray analysis of SVSP expression patterns in three cell lines  

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Table S2  List of T. parva SVSP genes, indicating the presence or absence of a signal peptide and/or nuclear localisation signal  

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Author Contributions  

Conceived and designed the experiments: JSM CC RB AP DAD. Performed the experiments: JSM CC SS SA IP SK JM. Analyzed the data: JSM CC VMN RB DAD. Contributed reagents/materials/analysis tools: JSM FK DM RB AP. Wrote the paper: DAD. 

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