Identification and characterisation of a *Theileria annulata* proline-rich microtubule and SH3 domain-interacting protein (TaMISHIP) that forms a complex with CLASP1, EB1, and CD2AP at the schizont surface

Sandra Huber | Tulin Karagenc | Dominic Ritler | Sven Rottenberg | Kerry Woods

1 Institute for Animal Pathology, Vetsuisse Faculty, University of Bern, Bern, Switzerland
2 Department of Parasitology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydin, Turkey
3 Institute of Parasitology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

Correspondence
Kerry Woods, Institute for Animal Pathology, Vetsuisse Faculty, University of Bern, Bern, Switzerland.
Email: kerry.woods@vetsuisse.unibe.ch

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Abstract

*Theileria annulata* is an apicomplexan parasite that modifies the phenotype of its host cell completely, inducing uncontrolled proliferation, resistance to apoptosis, and increased invasiveness. The infected cell thus resembles a cancer cell, and changes to various host cell signalling pathways accompany transformation. Most of the molecular mechanisms leading to *Theileria*-induced immortalization of leukocytes remain unknown. The parasite dissolves the surrounding host cell membrane soon after invasion and starts interacting with host proteins, ensuring its propagation by stably associating with the host cell microtubule network. By using BioID technology together with fluorescence microscopy and co-immunoprecipitation, we identified a CLASP1/CD2AP/EB1-containing protein complex that surrounds the schizont throughout the host cell cycle and integrates bovine adaptor proteins (CIN85, 14-3-3 epsilon, and ASAP1). This complex also includes the schizont membrane protein Ta-p104 together with a novel secreted *T. annulata* protein (encoded by TA20980), which we term microtubule and SH3 domain-interacting protein (TaMISHIP). TaMISHIP localises to the schizont surface and contains a functional EB1-binding SxIP motif, as well as functional SH3 domain-binding Px(P/A)xPR motifs that mediate its interaction with CD2AP. Upon overexpression in non-infected bovine macrophages, TaMISHIP causes binucleation, potentially indicative of a role in cytokinesis.

KEYWORDS

adaptor proteins, BioID, CD2AP, host–parasite interactions, microtubules, *Theileria*
We show that the schizont membrane protein Tainvolved in nuclear transport and constituents of the nuclear pore protein Jakmip1 at the parasite surface. We also show that proteins as parasite interactions at the parasite surface are a useful tool for discovering previously undescribed interactions, the kinetochore remains throughout the entire host cell cycle, and we showed that the MT apparatus, ensuring its distribution to both daughter cells (Hulliger, Wilde, Brown, & Turner, 1964; Schubert et al., 2010). Considering cytokinesis. Together, these data lead us to propose a role for TaMISHIP in regulating the interaction of the host cytoskeleton with the parasite.

2 | RESULTS

2.1 | Use of BioID to identify proteins at the T. annulata schizont surface

Host–parasite interactions in Theileria-transformed leukocytes are poorly characterised, so we performed BioID with the aim of identifying protein–protein interaction networks that occur at the schizont surface. This approach works by fusing a promiscuous Escherichia coli biotin protein ligase (BirA*) to a protein of interest and expressing it in cells. Upon adding biotin to cell culture media, proteins in close proximity to the fusion protein are biotinylated and can be affinity purified using streptavidin coated beads and identified by mass spectrometry (Roux et al., 2012). We fused myc-BirA* to the kinetochore binding domain of CLASP1 (amino acids 1256–1538) that was found to be sufficient for parasite binding (Huber et al., 2017). The fusion protein was expressed in T. annulata infected cells (TaC12) by lentiviral transduction, resulting in myc-BirA*-CLASP11256–1538 expression in approximately 60% of the population. Immunofluorescence analysis (IFA) with anti–myc antibodies confirmed that the myc-BirA* tag did not disrupt recruitment of CLASP11256 to the parasite surface (Figure 1a, top panel). Following incubation of transduced cells in culture media containing 50-μM biotin, we used FITC-conjugated streptavidin to confirm that proteins at and near the parasite surface were indeed biotinylated (Figure 1a, bottom panel). In nontransduced cells, no specific biotinylation of the parasite surface could be observed (not shown). Because the biotinylation reaction results in covalent biotin attachment to proximal proteins, stringent lysis conditions can be employed to solubilise protein complexes prior to affinity purification. This is an advantage when dealing with poorly soluble proteins, such as those incorporated in the parasite membrane. To test if biotinylated proteins could be detected by Western blotting following cell lysis, TaC12 myc-BirA*-CLASP11256–1538 cells were incubated with biotin, lysed exactly as described in (Roux et al., 2012), and probed with HRP-conjugated streptavidin. Lysates from nontransduced cells, or TaC12 myc-BirA*-CLASP11256–1538 cells cultured without additional biotin (not shown), served as negative controls. Biotinylated proteins of approximately 80 and 130 kDa were identified in the control experiment, which likely represent endogenously biotinylated proteins such as pyruvate carboxylase (130 kDa), biotinylated subunits of β-methyl-crotonyl-CoA carboxylase (80 kDa), or propionyl-CoA carboxylase (72 kDa; Chapman-Smith &
In TaC12_myc-BirA*–CLASP11256–1538 cells cultured in the presence of biotin, several additional biotinylated proteins could be detected (Figure S1A). A prominent band of approximately 70 kDa corresponds to the myc-BirA*–CLASP11256–1538 fusion protein, unsurprising seeing as BirA* is reported to biotinylate itself (Roux et al., 2012).

To facilitate mass spectrometry identification of biotinylated proteins, $2.5 \times 10^8$ transduced and nontransduced TaC12 cells were incubated with 50-μM biotin, subjected to stringent lysis, and affinity purified by using streptavidin coated beads. Proteins were digested on beads as described in (Roux et al., 2012) and analysed by mass spectrometry. By using this approach, we identified 2 Theileria
proteins (encoded by TA08425 and TA03615) with 5 and 10 unique peptides, respectively, and with no peptides identified in the negative control (Table 1 and S1). TA08425 encodes the schizont membrane protein Ta-p104, which we previously showed to interact with host EB1 via an SxIP motif (Woods et al., 2013). TA03615 encodes a hypothetical protein, which is predicted to be secreted and also contains an SxIP-motif and one FAINT (“frequently associated in Theileria”) domain. We recently confirmed that, like Ta-p104, TA03615 is expressed on the schizont surface and that both TA03615 and Ta-p104 interact (directly or indirectly) with CLASP1 (Huber et al., 2017).

### 2.2 MAPs and nuclear pore complex proteins localise close to the parasite surface

In addition to the two *Theileria* proteins, we also identified 18 bovine proteins as potential CLASP1<sub>1256-1538</sub> proximal proteins that were

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**TABLE 1** *Theileria annulata* and bovine proteins identified with confidence in the myc-BirA<sup>*</sup>-CLASP1<sub>1256-1538</sub> experiment

| ID      | Description                                                                 | Cellular location (function)                                                                 | %Coverage | #PSM | Protein Mass (Da) | TaC12<sub>myc-BirA<sup>*</sup>-CLASP1<sub>1256-1538</sub> | Number of unique peptides |
|---------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|-----------|------|------------------|----------------------------------------------------------|--------------------------|
| Q4UCH5  | *T. annulata* putative uncharacterized protein (TA03615)                     | Secreted, host cell cytoplasm/schizont membrane                                               | 19.51     | 17   | 90499.741        | 10                                                      |                          |
| L0 L373 | *T. annulata* major schizont surface protein p104 (TA08425)                 | Secreted, schizont plasma membrane                                                            | 10.26     | 20   | 101040.604       | 5                                                       |                          |
| E1BGU2  | CLASP1                                                                      | Cytoplasm (MT plus-end tracking protein, promotes stabilisation of dynamic MTs)             | 5.62      | 49   | 162254.491       | 8                                                       |                          |
| E1BQ15  | CLASP2                                                                      | Cytoplasm (MT plus-end tracking protein, promotes stabilisation of dynamic MTs)             | 1.88      | 23   | 168182.483       | 1                                                       |                          |
| E3W9A2  | CAP-Gly domain-containing linker protein 1 (CLIP-170)                       | Cytoplasm (MT plus-end tracking protein)                                                      | 3.92      | 2    | 160624.438       | 2                                                       |                          |
| A6QR54  | Janus kinase and microtubule-interacting protein 1 (Jakmip1)                | cytoplasm (binds to MTs and Jak family members)                                              | 3.51      | 2    | 73271.288        | 2                                                       |                          |
| Q28046  | Adseverin                                                                   | Cytoplasm (Ca<sup>2+</sup>-dependent actin filament severing protein)                       | 22.38     | 107  | 80577.919        | 8                                                       |                          |
| H7BWW0  | Coronin 1B                                                                  | Cytoplasm (actin filament nucleation, inhibition of actin filament depolymerization)        | 18.24     | 19   | 54252.78         | 5                                                       |                          |
| P79136-2| F-actin-capping protein subunit β (isoform β-2) (CapZB)                     | cytoplasm (blocking exchange of subunits at barbed ends of actin filaments)                 | 20.22     | 10   | 30628.743        | 3                                                       |                          |
| Q5E9F5  | Transgelin-2                                                                 | Cytoplasm (actin cytoskeleton)                                                               | 20.1      | 7    | 22426.497        | 2                                                       |                          |
| F1MDH3  | Talin-1                                                                     | Cytoplasm (connects actin filaments with integral membrane proteins)                        | 3.53      | 4    | 270815.461       | 3                                                       |                          |
| P19803  | Rho GDP-dissociation inhibitor 1 (Arhgdia)                                   | Cytoplasm (regulation of small GTPases)                                                      | 45.59     | 8    | 23421.4          | 4                                                       |                          |
| F1MM14  | CD2-associated protein (CD2AP, CMS)                                         | Cytoplasm (actin binding, adaptor protein)                                                   | 46        | 85   | 71203.755        | 23                                                      |                          |
| F1MNV9  | SH3 domain-containing kinase-binding protein 1 (CIN85)                     | Cytoplasm (adaptor protein)                                                                  | 5.09      | 8    | 68540.403        | 2                                                       |                          |
| E1B7Z1  | Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1 (ASAP1) | cytoplasm (GAP activity, phosphorylated by Src kinase, adaptor protein)                      | 5.05      | 7    | 123079.556       | 2                                                       |                          |
| G3NO16  | Crk-like protein (CRKL) (predicted)                                         | Cytoplasm (signal transduction)                                                              | 12.99     | 4    | 33386.587        | 3                                                       |                          |
| F1MFD5  | Ran GTPase-activation protein 1 (RanGAP) (predicted)                        | Cytoplasm, nuclear pore complex (stably interacts with RanBP2)                              | 12.31     | 8    | 62942.186        | 3                                                       |                          |
| F1MXS1  | E3 SUMO-protein ligase RanBP2                                               | Interphase: nuclear pore complex (mRNA export) Mitosis: centromere (important for chromosome segregation). | 38.58     | 208  | 336780.654       | 1                                                       | 72                        |
| F1MH31  | nuclear pore complex protein Nup214 isoform (predicted)                     | Nucleus (nuclear pore complex), cytoplasmic in mitosis                                       | 4.87      | 7    | 211599.748       | 5                                                       |                          |
| E1BW0   | E3 ubiquitin-protein ligase UBR1                                            | Cytoplasm (protein ubiquitination)                                                            | 2.81      | 6    | 199466.17        | 3                                                       |                          |

Note. Two *T. annulata* proteins (TA03615 and TA08415) were identified by mass spectrometry that were absent from the control. Seventeen bovine proteins were identified with one or more peptide hit in the myc-BirA<sup>*</sup>-CLASP1<sub>1256-1538</sub> sample that were absent from the control. One bovine protein (FIMXS1) was identified by 72 peptides, with one peptide identified in the control. Uniprot and GeneDB were used to assign accession numbers (ID), description, and cellular localisation. The %coverage value indicates the percentage of the protein that is covered by the peptides identified by the mass spectrometry analysis. PSM is peptide spectral match, and #PSM reflects the total number of identified peptides for one particular protein. The #peptides column lists the number of different peptides found for each protein. MT = microtubule.
not identified (or in the case of one protein, identified with only one peptide) in the negative control (Table 1 and S1). Because proximity-dependent labelling does not discriminate between true interaction partners and proteins that come into transient proximity with the bait, we tested the localisation of identified proteins by IFA. Among the proteins found were several MAPs, including CLASP1 itself, CLASP2, CAP-Gly domain-containing linker protein 1 (CLIP-170), and Janus kinase and MT-interacting protein 1 (Jakmip1). The presence of endogenous CLASP1 and CLASP2 validated our approach, as we have already shown that both proteins associate with the schizont surface (Huber et al., 2017). Jakmip1 interacts with both MTs and members of the Jak family (Jak1 and Tyk2; Steindler et al., 2004). Analysis of endogenous Jakmip1 in TaC12 cells revealed an association with MTs as reported, and in many cells, the outline of the parasite was labelled with Jakmip1 (Figure 1b). TaC12 cells transiently transfected with Jakmip1-V5 confirmed this observation, with the fusion protein localising to the centrosomes and along the length of MTs, with some weak accumulation along the parasite membrane (not shown). On the other hand, expression of GFP-CLIP-170, a well characterised MT + TIP (plus-end tracking protein), revealed no convincing association with the parasite surface (data not shown). CLIP-170 interacts with the C-terminal EEY-motif of EB1 via its CAP-Gly domain (Honnappa et al., 2006), with the C-terminal (kinetochore-binding) domain of CLASP1 (Akhmanova et al., 2001), and with tubulin dimers (Folker, Baker, & Goodson, 2005). Considering the association of EB1, CLASP1, and tubulin with the schizont surface, it is likely that CLIP-170 comes into transient contact with the parasite and was therefore identified in our search. Several proteins involved in actin cytoskeleton regulation (Adseverin, Coronin 1B, CapZB, Transgelin-2, Talin-1, and Rho GDP-Dissociation Inhibitor [Arhgdia]) were also identified in our CLASP11256–1538 BioID experiment. We tested the localisation of Talin-1 and Coronin 1B in parasitised cells and found that upon overexpression neither GFP-Talin1 nor mCherry-Coronin 1B exhibited any convincing parasite localisation (not shown), suggesting that their identification in the BioID pull down is likely due to their abundance in the cytoplasm rather than a specific interaction.

RanGAP1 (Ran GTPase-activating protein 1) and RanBP2 (Ran binding protein 2, also known as Nup358) were also identified in our BioID experiment. These proteins are associated with one another throughout the cell cycle, localising to the nuclear pore complex during interphase, and to kinetochores and the mitotic spindle during mitosis where they are involved in sister chromatid segregation (Joseph, Tan, Karpova, McNally, & Dasso, 2002; Matunis, Wu, &Blobel, 1998; Werner, Flotho, & Melchior, 2012). Indirect IFA revealed that both proteins localise to TaC12 host cell nuclear membranes during interphase as reported and also accumulate close to schizont membranes in a pattern that resembles parallel lines or sheet like structures (Figure 1c, top and middle panels). GFP-RanGAP1 was also found to localise in the vicinity of the schizont upon overexpression in TaC12 cells (not shown), confirming our observations made with the endogenous protein. The nuclear pore complex is a large structure that forms pores in the nuclear envelope, facilitating the selective transport of macromolecules between the nucleus and the cytoplasm. It is an assembly of several nuclear pore complex components (Nups), including Nup214, which is localised to the cytoplasmic face of the nuclear pore complex in interphase cells and is required for correct cell cycle progression and nuclear-cytoplasmic transport (van Deursen, Boer, Kasper, & Grosfeld, 1996). Nup214 was identified in the CLASP11256–1538 BioID experiment with five unique peptides, and we found that GFP-Nup214 localised to the nuclear pore in TaC12 cells as expected and close to the schizont surface in a similar pattern as RanGAP1 and RanBP2 (Figure 1c, bottom panel).

### 2.3 The adaptor proteins CD2AP, CIN85, and ASAP1 are detected at the schizont surface

In addition to the MAPs and the nuclear pore complex proteins, a number of adaptor proteins, namely, CD2-associated protein (CD2AP), SH3 domain-containing kinase-binding protein 1 (c-Cbl-interacting protein of 85 kDa, CIN85), Arf-GAP with SH3 domain ANK repeat and PH domain-containing protein 1 (ASAP1), and Crk-like protein (CrkL), were identified. Adaptor proteins are molecules possessing two or more protein-binding domains that function to facilitate the formation of large signalling complexes. This allows them to play pivotal roles in signal transduction, while usually possessing no catalytic activity themselves (Flynn, 2001). One prominent example is Crk (chicken tumour virus no. 10 regulator of kinase), an oncogene with the ability to transform cells. Crk consists of Src homology 2 (SH2) and SH3 protein–protein interaction domains but no catalytic domain (Mayer, Hamaguchi, & Hanafusa, 1988; reviewed in Flynn, 2001; Akagi, Murata, Shishido, & Hanafusa, 2002). Our BioID experiment identified CrkL as a possible parasite interacting protein, but GFP-tagged CrkL and the related Crk failed to co-localise significantly with the parasite (not shown). On the other hand, we could confirm that both endogenous ASAP1 (Figure 2a, top panel) and overexpressed tagged ASAP1 (not shown) localised close to the schizont surface. ASAP1 is known to constitutively bind to CIN85 (Kowanetz et al., 2004) and functions as a GTPase-activating protein (GAP) for Ras-related small GTPases (Brown et al., 1998).

The CIN85 and CD2AP/CMS family of adaptor molecules are ubiquitously expressed and have been implicated in multiple protein-protein interaction networks and signal transduction pathways (Dustin et al., 1998; Take et al., 2000; Watanabe et al., 2000; reviewed in Dikic, 2002). We investigated the localisation of CD2AP, for which 23 unique peptides were found, in Theileria-infected cells. In paraformaldehyde (PFA) fixed cells, CD2AP is always found at the schizont surface (Figure 2a, middle panel). The anti-CD2AP antibody specificity was tested in IFA with non-infected BoMac. In BoMac cells CD2AP accumulates in the cytoplasm and the leading edge of migrating cells (not shown). This localisation of CD2AP in bovine cells is comparable with its localisation in Cos7 cells, where it is found in the cytoplasm, membrane ruffles, and the leading edges of migrating cells (Kirsch, Georgescu, Ishimaru, & Hanafusa, 1999). To exclude that CD2AP localisation in fixed cells is an artefact, and to analyse the dynamics of CD2AP localisation throughout the cell cycle, TaC12 cells were transduced with a GFP-CD2AP construct. Live cell imaging revealed that overexpressed GFP-CD2AP localises to the schizont surface throughout the cell cycle (Movie S1). These properties make CD2AP the second host cell protein that we identified which exclusively...
localises to the *Theileria* schizont surface throughout the host cell cycle. CLASP1 shows a very similar behaviour (Huber et al., 2017), whereas the interaction of other host cell proteins with the schizont varies throughout the cell cycle (e.g., PLK1, Schubert et al., 2010, and EB1, Woods et al., 2013), or depend on the presence of MTs (e.g., CLASP2, Huber et al., 2017). CIN85 shares significant structural similarity with CD2AP. Therefore, we performed the same analyses with CIN85, for which two peptides were found in the BioID experiment. Like CD2AP, endogenous CIN85 accumulates close to the schizont surface (Figure 2a, bottom panel), and its localisation to the schizont throughout the entire cell cycle was confirmed by live cell imaging of GFP-CIN85 expressing cells (Movie S2). Although CIN85 has previously been observed in a diffuse pattern in both the cytoplasm and the nucleus in some cells (e.g., in the human prostate carcinoma [PC-3 U] cells; Yakymovych et al., 2015), we think it is likely that the small amount of CIN85 and CD2AP observed in the nucleus of TaC12 cells is an artefact due to non-specific antibody labelling, especially considering that ectopically expressed CD2AP/CIN85 was not found in the nucleus.

### 2.4 Use of CD2AP and CIN85 to identify further protein interaction networks at the schizont surface by using BioID

Because CD2AP and CIN85 have been implicated in multiple protein-protein interaction networks and signal transduction pathways (reviewed in Dikic, 2002), we asked the question if they could contribute to *Theileria*-induced transformation by recruiting signalling molecules to the schizont surface. CD2AP has been shown to bind to the tumour suppressor protein p53 in the cytoplasm of human lung carcinoma cells (Panni et al., 2015), and considering that p53 has been

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**FIGURE 2** Analysis of bovine adaptor protein (ASAP1, CD2AP, and CIN85) localisation, and function of myc/BirA*-CD2AP. (a) TaC12 cells were stained with anti-ASAP1 (green, top panel), anti-CD2AP (green, middle panel) or anti-CIN85 (green, bottom panel). The parasite was labelled with anti-p104 (red), and host and parasite nuclei were labelled with DAPI (blue). (b) TaC12 cells were transduced with the myc/BirA*-CD2AP construct and analysed by immunofluorescence microscopy. Cells were stained with anti-myc (green), and the parasite was labelled with anti-TaSP (red); DNA was labelled with DAPI (blue, top panel). TaC12_myc-BirA*-CD2AP cells were incubated with 50-μM biotin and subsequently stained with FITC conjugated streptavidin (green). The schizont was labelled with anti-TaSP (red), and DNA was labelled with DAPI (blue, bottom panel). Scale bar = 10 μm.
reported to be sequestered at the surface of the Theileria schizont (Haller et al., 2010), we hypothesised that schizont-associated CD2AP could play a role in recruiting p53 to the parasite. To test this, we first expressed short hairpin RNAs (shRNAs) in Theileria-infected macrophages to deplete bovine CD2AP and obtained a mixed population in which the majority of cells expressed no detectable CD2AP (Figure S2A). Following selection with puromycin, CD2AP was barely detectable by Western blot (Figure S2B). Selected cells displayed a 2-fold reduction in relative proliferation rate following 7 days in culture when compared to wild-type (WT) TaC12 cells or those transduced with GFP-CD2AP, or a non-CD2AP-targeting shRNA control plasmid (Figure S2C). To address our hypothesis that CD2AP functions to recruit signalling molecules to the parasite surface, we analysed the localisation of p53 and IKK in TaC12 cells following CD2AP depletion. Although the pattern of both p53 and IKK was rather heterogeneous in TaC12 cells (this has been previously described for IKK expression in TaC12 cells; Schmucki-Maurer et al., 2010), we could detect both proteins with similar patterns in close proximity to the parasite surface in both WT and CD2AP-depleted cells (Figure S2D). These data suggested that CD2AP is unlikely to be involved in recruiting either IKK or p53 to the parasite surface. It is possible that the depletion of CD2AP achieved with shRNA expression was insufficient to abolish the recruitment of signalling molecules to the parasite: however, our attempts to use CRISPR/Cas9 to knock out CD2AP from infected macrophages failed with two guide RNA combinations. Wang et al. (2015) report that CD2AP is essential in human cancer cell lines, and we cannot exclude that CD2AP also plays an essential role in Theileria-transformed cells. To further address the possibility that CD2AP and/or CIN85 are involved in forming signalling complexes at the parasite surface, we next used both adaptor proteins to target BirA* to the schizont surface and performed further BioID experiments. TaC12 cells were transduced with myc-BirA*-CD2AP or myc-BirA*-CIN85 constructs and selected with G418. The ability of the adaptor proteins to target functional myc-BirA* fusion proteins to the schizont surface was confirmed by IFA with anti-myc antibodies (Figure 2b, top panel, not shown for myc-BirA*-CIN85) and by staining with FITC-conjugated streptavidin (Figure 2b, bottom panel, not shown for myc-BirA*-CIN85). In both cell lines, biotinylated mainly occurred at the schizont surface, and only a slight staining in the host cell cytoplasm could be detected. After these preliminary tests the cells were grown and prepared for lysis followed by pull down with streptavidin coated beads, as described above for CLASP11256-1538. We identified 44 bovine proteins in the myc-BirA*-CD2AP sample with no peptides in the control sample, and 21 proteins in the myc-BirA*-CIN85 sample when excluding highly abundant ribosomal and histone proteins (Table S2). A number of host proteins were identified in all three BioID experiments, including CLASP1, CD2AP, CIN85, ASAP1, and RanBP2.

In addition to the three nuclear pore complex proteins identified in the CLASP11256-1538 BioID experiment, the CD2AP BioID pull down identified Nup160 and three Importins (Importins A, B1, and 5)—four more proteins that are involved in transport between the nucleus and cytoplasm. Nup160 is part of the nuclear pore “Y-complex” and is localised both to the cytoplasmic and nuclear face of NPCs where it functions as a major scaffold part of the pore (Morchoisne-Bolhy et al., 2015). Importins bind to cargo proteins in the cytoplasm, and the complex is transported through the nuclear pore into the nucleus (reviewed in Kubitscheck & Siebrasse, 2017). Transient transfection of TaC12 cells revealed that both GFP-Nup160 and GFP-Importin B1 localise close to the schizont surface, as well as to the nuclear membrane or nucleus as expected (Figure S1B). The pattern of these two proteins at (or near) the schizont surface is comparable to the staining pattern of RanGAP1, RanBP2, and Nup214 (compare Figures 1c and S1B). Host and parasite proteins identified in the three BioID experiments are summarised in a Venn diagram in Figure 3.

### 2.5 CD2AP, Ta-p104, and TaMISHIP (TA20980) interact at the T. annulata schizont surface

The BioID pull down using myc-BirA*-CD2AP as a bait identified two T. annulata proteins Ta-p104 (encoded by TA08425) and one encoded by TA20980. Although we have already described some functions of Ta-p104 (Huber et al., 2017; Woods et al., 2013), there is little information available for TA20980. TA20980 encodes a protein of 988 amino acids that contains a signal peptide of 19 amino acids with a cleavage probability of 98.2%. We propose naming the product encoded by TA20980 “TaMISHIP” (T. annulata proline-rich microtubule and SH3 domain-interacting protein), because we found that it can track growing MT plus ends and contains functional SH3-binding motifs (see below). A blast search on http://blast.ncbi.nlm.nih.gov identified a homologous protein with a sequence identity of 68% in Theileria lesotocardi (DQ00498) and one with a sequence identity of 42% in Theileria parva (TP01_0380; Figure S3). Interestingly, no homologue could be found in the nontransforming T. orientalis genome, making TaMISHIP a candidate for being a transforming factor (Hayashida et al., 2012). The T. lesotocardi homologue was isolated as an immunogenic protein (“Clone 5”) that localises to the schizont surface and is expressed in two splice variants (Bakheit, Scholzen, Ahmed, & Seitzer, 2006). The two splice variants were also found in T. annulata and T. parva, although a function or life cycle specific expression of one or the other variant has not yet been described (Bakheit, Ahmed, & Seitzer, 2008). Microarray data shows that TA20980 is expressed in all life cycle stages, exhibiting a slight increase in gene expression in the schizont stage, with a gradual decrease through merozoites to piroplasms (Shiels and Weir, unpublished). Indirect IFA in TaC12 cells revealed that endogenous TaMISHIP is localised at the schizont surface (Figure 4a). Although TaMISHIP contains several nuclear localisation signals, and no domains that would suggest integration into the schizont membrane, we did not detect TaMISHIP in the host nucleus. We conclude that TaMISHIP is most likely secreted and then binds to other schizont surface proteins, although we cannot fully exclude the possibility that the endogenous protein is exported to the nucleus in amounts that are below the detection threshold. Analysis of TaMISHIP localisation in freshly invaded peripheral blood mononuclear cells indicates that it co-localises with Ta-p104 in sporozoites, likely in the microneme/rhoptry complex (Iams et al., 1990), and translocates to the surface of the parasite as it differentiates into a schizont (Figure S4A). Recently, it was reported that CD2AP is involved in Toxoplasma gondii invasion (Guérin et al., 2017), and so we also tested the localisation of bovine CD2AP following infection...
with *Theileria* sporozoites (Figure S4B). We could detect no convincing accumulation of CD2AP in the early stages of infection, suggesting that CD2AP is unlikely to play a role in invasion. We could first detect CD2AP close to the parasite surface after 24 hr when differentiation into the schizont stage had started, suggesting that CD2AP recruitment occurs only after the parasite establishes its intracellular niche.

The TaMISHIP protein sequence includes two EB1-binding SxIP motifs, three putative SH3-binding Px(P/A)xPR motifs, two regions consisting of several nuclear localisation signals, and one nuclear export signal (data from elm.eu.org; Figure 4b). Although the sequence homology between the *T. annulata* protein and the corresponding *T. parva* and *T. lestoquardi* proteins is rather low, these domains are conserved in all three species (Figure S3). Px(P/A)xPR motifs can mediate interactions with SH3 domains (Kowanetz et al., 2003), and this motif is found 3 times in TaMISHIP (Figure 4b, Figure S3). Because CD2AP and CIN85 possess three SH3 domains each, an interaction of TaMISHIP with CD2AP and/or CIN85 is possible. This was tested by immunoprecipitating (IP) endogenous CD2AP, CIN85, and TaMISHIP from *Theileria*-infected cells. Co-precipitated proteins were identified by mass spectrometry, and for selected proteins, the interaction was confirmed by Western blotting. CD2AP was found in TaMISHIP-IPs, and vice versa, indicating that TaMISHIP and CD2AP interact with one another either directly or indirectly. We also detected four EB1 peptides by mass spectrometry and a faint band by Western blot following IP of TaMISHIP, indicating a weak interaction between these two molecules is not surprising and is similar to what we have previously described for Ta-p104–EB1 interaction (Woods et al., 2013). Ta-p104 and bovine CLASP1 were found repeatedly in both IPs. Together, these data suggest that Ta-p104, CLASP1, and EB1 are part of the TaMISHIP–CD2AP complex (Figures 4c,d, S5, and S6). CIN85 was also identified by mass spectrometry in both IPs, although we could not confirm this by Western blotting. The IP of
FIGURE 4  Endogenous TaMISHIP localises to the Theileria schizont surface and interacts with CD2AP in a protein complex on the Theileria annulata schizont surface. (a) Endogenous TaMISHIP was stained with anti-TaMISHIP (green), and the parasite was labelled anti-p104 (red). Host and parasite nuclei were labelled with DAPI (blue). Scale bar = 10 μm. (b) TaMISHIP protein structure showing different important domains of the T. annulata protein. The protein consists of 970 amino acids and comprises two EB1-binding SxIP motifs (yellow), several domains with nuclear localisation signals (NLS, green), a domain with a nuclear export signal (NES, blue), and SH3 domain-binding Px(P/A)xPR motifs (orange). (c) TaC12 cells were lysed and subjected to co-immunoprecipitation (co-IP) with rabbit polyclonal anti-CD2AP or rabbit IgG (control) antibodies. For each sample the nonsoluble pellet (P), lysate supernatant (SN), IP flow through (FT; for each 1.5% of total amount), and 3.3% of total bound fraction after IP (IP) was analysed by SDS-PAGE. Analysis with anti-CD2AP antibodies confirmed the successful immunoprecipitation of CD2AP. The co-precipitation of the parasite proteins TaMISHIP and Ta-p104, and the host cell protein CLASP1 could be confirmed with the corresponding antibody probes. (d) TaC12 cells were lysed and subjected to co-immunoprecipitation (co-IP) with rat polyclonal anti-TaMISHIP or rat IgG (control) antibodies. As a control, the anti-TaMISHIP immunoprecipitation was also performed with non-infected BoMac that do not express the T. annulata protein TaMISHIP. For each sample, the nonsoluble pellet (P), lysate supernatant (SN), IP flow through (FT; for each 1.5% of total amount), and 5% of total bound fraction after IP (IP) was analysed by SDS-PAGE. Analysis with anti-TaMISHIP antibodies confirmed the successful immunoprecipitation of TaMISHIP. The co-precipitation of host cell CD2AP, Ta-p104, CLASP1, and EB1 could be confirmed with the corresponding antibody probes. (e) HEK 293T cells transfected with WT GFP-TaMISHIP or GFP-TaMISHIP in which all three Px(P/A)xPR motifs were mutated to Px(P/A)xPA (GFP-TaMISHIP3XKPR➔KPA) were lysed 16 hr after transfection and subjected to co-IP using GFP-TRAP magnetic beads. For each sample, the nonsoluble pellet (P), lysate supernatant (SN), IP flow through (FT; for each 1% of total amount), and 10% of total bound fraction after IP (IP) was analysed by Western blotting. Analysis with anti-eGFP (enhanced GFP) antibodies confirmed the expression of the fusion proteins and their precipitation with GFP-TRAP beads. GFP-TaMISHIP is predicted to run at around 136 kDa, and the additional band detected with the anti-GFP antibody might be degradation products of the fusion protein. Although CD2AP is co-precipitated with WT GFP-TaMISHIP, no interaction can be seen between mutated GFP-TaMISHIP and CD2AP.
CIN85 did not, however, co-precipitate CD2AP or TaMISHIP (Figure S7). These data might indicate that CIN85 associates rather weakly with the CD2AP/CLASp1/TaMISHIP/Ta-p104 complex. We also found several isoforms of the adaptor protein 14-3-3 in both CD2AP and TaMISHIP IPs. Like CD2AP and CIN85, 14-3-3 family proteins can interact with multiple proteins—170 associated proteins that play roles in signal transduction or cellular communication have been identified for 14-3-3 gamma (Jin et al., 2004), and like CD2AP and CIN85, 14-3-3 epsilon is clearly recruited to the vicinity of the schizont surface (Figure S1C). The proteins identified by mass spectrometry following CD2AP, TaMISHIP, and CIN85 IPs are summarised in a Venn diagram (Figure S8), and the raw data can be found in Tables S3 and S4. Structured illumination microscopy on a DeltaVision OMX Blaze system revealed little co-localisation of CD2AP, TaMISHIP, and Ta-p104, although they are found to localise very close to each other at the schizont surface (Figure S9, 1 pixel = 40 nm). Another method used to confirm the close localisation of proteins is the proximity ligation assay (Duolink) that generates a fluorescent signal when two proteins are in very close proximity (less than 40 nm). This assay yields a signal for the combinations CD2AP-TaMISHIP and CD2AP-Ta-p104, confirming the close proximity and likely interaction (direct or otherwise) of these proteins at the schizont surface. As a positive control, Duolink was performed with CLASp1 and Ta-p104, a known interacting pair (Huber et al., 2017; Figure S10). To test whether the Px(P/A)xPR motifs found in TaMISHIP are specifically responsible for recruiting CD2AP to the schizont surface, we mutated all three motifs to Px(P/A)xPA, expressed GFP-fusion proteins in HEK 293T cells, and subjected lysates to IP using GFP-nanobody coated beads (GFP-Trap).

We repeatedly noticed that in the presence of WT GFP-TaMISHIP, endogenous CD2AP was poorly soluble and was found more abundantly in the pellet (insoluble) fraction after lysis when compared to cells expressing mutated GFP-Ta20980 (Figure 4e) or control cells not expressing any fusion protein (not shown). This could indicate that TaMISHIP forms a complex with CD2AP that leads to reduced solubility. Importantly, while WT GFP-TaMISHIP co-precipitated CD2AP, no interaction of the mutated protein (GFP-TaMISHIP<sub>R173A/R189A/R205A</sub>) with CD2AP could be detected by Western blot (Figure 4e, Figure S7D). These results confirm that the interaction between TaMISHIP and CD2AP is specific and is mediated by the Px(P/A)xPA motifs contained within TaMISHIP.

### 2.6 TaMISHIP can track MT plus ends and causes binucleation upon overexpression

Because TaMISHIP possesses two SxIP motifs, we considered that a localisation to growing MT plus ends is possible. We had previously tested whether the two SxIP motifs found within TaMISHIP were sufficient to mediate MT plus-end tracking by separately expressing fragments containing one or the other of the SxIP motifs in COS7 cells, and analysing their localisation following fixation. This approach failed to reveal any interaction with MT plus ends (Woods et al., 2013). Now, to investigate the potential interaction of TaMISHIP with MT plus ends more fully, we tested the ability of full length GFP-TaMISHIP to track MT plus ends by imaging live BoMac cells with a highly sensitive microscope. The resulting time lapse video shows that GFP-TaMISHIP accumulates in the nucleus and the cytoplasm during interphase and that it can indeed track MT plus ends (Figure 5a, Movie S3). The discrepancy with our previous results might be caused by fixation, seeing that full-length TaMISHIP was found to localise to MT plus ends only by live cell imaging. These observations could also indicate that the conformation of the full-length protein is important for plus-end tracking. Although mutation of the first SxIP motif (SPIP, aa 183–186) to SPNN did not abolish TaMISHIP localisation to MT plus ends (Movie S4), the mutation of the second motif (SKIP, aa 796–799) to SKNN completely prevented localisation of GFP-TaMISHIP to MT plus ends (Movie S5), indicating that this motif is necessary to mediate MT plus-end tracking. Live cell imaging as cells progressed through mitosis revealed that TaMISHIP associates with the mitotic spindle, providing further evidence of an interaction with MTs (Figure 5b, Movie S6). Finally, we observed a 2.5-fold increase in binucleation 24 hr post transfection (Figure 5c,d, Movie S7), indicating a potential involvement of TaMISHIP in mitotic progression or cytokinetic abscission.

### 3 DISCUSSION

In this work, we have discovered a core network of interacting proteins, including CLASp1, EB1, CD2AP, Ta-p104, and TaMISHIP (TA20980), that coats the Theileria surface throughout the cell cycle, and which likely plays a role in mediating interactions between the parasite and the host cytoskeleton. We also demonstrated the presence of nuclear pore complex proteins close to the surface of the schizont. This was achieved by employing BioID technology (Roux et al., 2012). BioID has been successfully used in parasitology to identify novel components of the Trypanosoma brucei cytoskeleton (McAllaster et al., 2015; Morriswood et al., 2013), the Toxoplasma gondii inner membrane complex (Chen et al., 2015), and dense granules (Nadipuram et al., 2016), as well as proteins with possible functions in gametocyte egress in Plasmodium (Kehrer, Frischknecht, & Mair, 2016). This is the first time that it has been applied to Theileria-infected cells. We confirmed the association of 10 bovine proteins with the schizont (CD2AP, CIN85, ASAP1, 14-3-3 epsilon, Jakmip1, RanGAP1, RanBP2, Importin B1, Nup214, and Nup160) and show that CD2AP and CIN85 interact in a complex with CLASp1, EB1, 14-3-3 epsilon, and two T. annulata surface proteins (Ta-p104 and TaMISHIP). Figure 6 summarises our current understanding of protein interactions that occur at the schizont surface.

We were particularly intrigued at the presence of several adaptor proteins at the host–parasite interface. Adaptor proteins are known to recruit signalling proteins into complexes, which led us to speculate that the schizont recruits these proteins to initiate signalling cascades on its surface. CD2AP and CIN85 share significant sequence and structural similarity and are comprised of three N-terminal SH3 domains, a C-terminal proline-rich domain, multiple phosphorylatable residues, and a coiled-coil domain that mediates homodimerisation. Further, CD2AP possesses four actin-binding domains. Multiple binding partners have been described for CD2AP and CIN85, and they have been implicated in diverse biological processes including regulating actin cytoskeleton dynamics (Bruck et al., 2006; Hutchings, Clarkson, Chalkley, Barclay, & Brown, 2003; Kirsch et al., 1999; Zhao et al.,...
Live cell imaging was performed with BoMac cells expressing GFP-TaMISHIP, and pictures were taken every 2 s. The top panel shows a single time point from Movie S3. The region magnified in three subsequent time points (bottom panels) is indicated with a white rectangle. MT plus-end tracking behaviour of GFP-TaMISHIP is indicated with arrow heads. Scale bar = 5 μm. (b) Live cell imaging was performed with BoMac cells expressing GFP-TaMISHIP, and pictures were taken every 10 min. The top panel shows a BoMac cell just prior to mitosis, when GFP-TaMISHIP accumulates in the nucleus. The middle and bottom panels show the progression through mitosis, and the accumulation of GFP-TaMISHIP on the mitotic spindle. The corresponding live cell imaging can be found in Movie S4. Scale bar = 5 μm. (c) TaMISHIP-V5 was overexpressed in BoMac cells, and fixed cells were stained with anti-V5 (green). DNA was labelled with DAPI (blue). Scale bar = 10 μm. (d) BoMac cells were transfected with TaMISHIP-V5 and fixed 24 hr after transfection. Binucleation was counted in nontransfected and transfected cells. Shown is the mean relative number of binucleated cells in three independent experiments (n = 131, 90, 89; p = .0031).
of a large network of (directly or indirectly) interacting proteins at the schizont surface that mediate interactions between the schizont and the host cytoskeleton and are important for regulating cytokinesis.

One specific aim of our study was to identify novel parasite molecules with the potential to interact with the host. We identified 12 T. annulata proteins in our co-IP experiments, after excluding the highly abundant and intracellular ribosomal proteins. These proteins are summarised and described in Table S5. In order for a Theileria protein to qualify as a potential “host cell manipulator,” several criteria must be fulfilled. The candidate proteins should be uniquely expressed in transforming Theileria species, and absent from the nontransforming species T. orientalis or other nontransforming apicomplexans such as Toxoplasma or Plasmodium. The protein should be expressed in the schizont stage, as only this stage is associated with host cell transformation, and finally, the protein should be expressed at the parasite surface or secreted into the leukocyte cytosol (Shiels et al., 2006). Of the 12 proteins we identified in our experiments, only TaMISHIP fulfils these strict criteria and was chosen for further study, and indeed an attempt to use bioinformatics screening to identify potential manipulators of the host phenotype revealed TaMISHIP as a potential candidate (Hayashida et al., 2012; Shiels et al., 2006). We showed that TaMISHIP, like Ta-p104, interacts with CLASP1 (Huber et al., 2017) and EB1 (Woods et al., 2013) and can track MT plus ends in an XidP-motif dependent manner. This makes it likely that TaMISHIP plays a role in the regulation of MT dynamics at the parasite surface. The overexpression of TaMISHIP in non-infected cells led to an increase in binucleation. Considering its localisation at the mitotic spindle during mitosis, and its interaction with EB1, we propose that the most likely explanation for this is that TaMISHIP overexpression interferes with cytoskeleton dynamics and inhibits cytokinesis. On the basis of our data, we cannot exclude the possibility that the nuclear localisation of overexpressed TaMISHIP during interphase contributes to the binucleation phenotype. However, because we did not detect endogenous TaMISHIP in the nucleus of infected cells, but rather at the parasite surface, we find this rather unlikely. Taken together, our results point towards a function of the CD2AP/Ta-p104/TaMISHIP/CLASP1/EB1 complex in mediating the interaction of the schizont with host cell MTs.

In summary, we have identified several previously unexplored host–parasite interactions in Theileria-infected cells. Further work is now required to uncover other potential functions of parasite-associated adaptor proteins and to elucidate the role that nuclear pore complex proteins play in Theileria-leukocyte interactions.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell culture, transfections, transductions, and cell viability

T. annulata-infected macrophages (TaC12) were obtained by in vitro infection of BoMac and cultured in Leibovitz 15 medium (Gibco) containing 10% fetal calf serum (FCS, BioConcept). 10-mM HEPES pH 7.2 (Merck), 2-mM L-glutamine (Lonza), 70–μM β-mercaptoethanol (Merck), and antibiotics (Lonza) as described previously (Shiels et al., 1992). SV40-transformed BoMac and HEK 293T cells were cultured as described previously (Schubert et al., 2010). TaC12 and BoMac cells were electroporated by using Amaxa 4D Nucleofector (Lonza) with cell solution SF and the program DS103. For GFP fusion protein expression, HEK 293T cells were transfected with FuGENE® HD Transfection Reagent (Roche) following the manufacturer’s instructions. For lentiviral production, HEK 293T cells were transfected as described in (Huber et al., 2017). Media containing lentiviral particles was collected and used for transduction of TaC12 and BoMac cells. After transduction, cells expressing the gene of interest were selected by using the antibiotics G-418 sulfate (Promega) or puromycin (Gibco). To test cell viability in cells depleted of CD2AP or overexpressing GFP-CD2AP, 300 cells were seeded in 200-μL Leibovitz 15 medium in 96 well plates and cultured for 3 to 7 days at 37 °C. Viability was assessed by addition of 10 μL of a 0.5-mM solution of Resazurin (Sigma) to each well, followed by an incubation of 4 hr at 37 °C. Fluorescence intensity was measured on an EnSpire 2300 Multilabel Reader (PerkinElmer).

4.2 | Kits and antibody production

The in situ proximity ligation assay (Duolink®) was purchased from Sigma. The GST fusion proteins (GST-TaMISHIP300–400, GST-CIN85130–230, and GST-CIN85470–620) were expressed in BL21 Star E. coli (Invitrogen), purified by using glutathione sepharose beads (GE Healthcare), and sent to Eurogentec for antibody production and purification.

FIGURE 6 Graphical representation of host–parasite interactions on the Theileria annulata schizont surface. Parasite surface-associated proteins identified in our BioID and co-IP experiments (Ta-p104, gp34, TaMISHIP, and TA03615) are shown in grey. Microtubule-associating proteins found with BioID (CLASP1, Jakmip1), or previously published as binding to the parasite (EB1), are represented in green. Adaptor proteins (CD2AP, CIN85, ASAP1, and 14-3-3 epsilon) are represented in yellow, and proteins involved in nuclear transport (RanGAP1, RanBP2, Nup214, Nup160, and Importin B1) are represented in blue. The cell cycle-dependent association of Plk1 with the schizont and the recruitment of the IKK signalosome complex and p53 have been described previously, and P in a circle represents phosphorylation.
4.3 Primers, antibodies, and expression constructs

Primers, antibodies, and expression constructs are listed in Table S6.

4.4 IFA and time-lapse imaging

Cells were seeded and grown on coverslips and fixed with 4% PFA for 10 min at room temperature. Cells were permeabilised in 0.2% Triton X-100 and antibodies were diluted in 10% heat-inactivated FCS in PBS. DNA was stained with DAPI (Invitrogen), and samples were mounted by using mounting media (DAKO). Fixed samples and time-lapse experiments were analysed on a DeltaVision Elite High Resolution Microscope system (GE Healthcare) with Olympus IX-70 inverted microscope with a CMOS camera, 60× Olympus Objective, and SoftWorx (Applied Precision) software. Samples in Figures 1, 2 (top and bottom panels), S1B,C, and S2A,D were analysed on a Nikon Eclipse 80i wide-field microscope with a Hamamatsu Orca R2 camera using PlanApo objectives (Nikon) and the OpenLab 5 software (Improvision). Samples in Figure S9 were analysed on the DeltaVision OMX Blaze system (GE Healthcare) with sCMOS cameras at the Biozentrum in Basel, Switzerland. Fiji (ImageJ) software (Schindelin et al., 2012) and Photoshop (Adobe) were used to process the images.

4.5 Use of BioID to identify proteins proximal to and interacting with CLASP1\textsubscript{1256–1538}, CD2AP, and CIN85

The BioID approach was applied as described before (Roux et al., 2012). Briefly, TaC12 WT cells (control) and TaC12 cells expressing a myc-BirA\textsuperscript* fusion protein were incubated in media containing 50-µM biotin (Serva) and subjected to stringent lysis. Biotinylated proteins were affinity purified with 600-µl MyOne Streptavidin C1 Dynabeads (Invitrogen). Proteins were eluted from 10% of the beads for Western blot analysis, and the remaining 90% of the beads were subjected to on beads tryptic digest and analysis by mass spectrometry on an ESI-ion trap or QTOF mass spectrometer at the Proteomics and Mass Spectrometry Core Facility, Department of Clinical Research, University of Bern, Switzerland. HEK 293T cells transiently expressing GFP-TaMISHIP fusion proteins were lysed in lysis buffer containing 0.5% NP-40 and subjected to GFP-TRAP co-IP (Chromotek) following the manufacturer’s instructions.

4.6 Co-immunoprecipitation and mass spectrometry

Wild-type TaC12 and BoMac cells were lysed in modified RIPA buffer (50-mM Tris–HCl pH 7.4, 150-mM NaCl, 1-mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 2-mM sodium vanadate, 25-mM NaF, protease inhibitor cocktail [Roche]) and subjected to co-IP. For the IP of endogenous CD2AP in TaC12 cells, 12 µg of anti-CD2AP antibodies or 12 µg of IgG from rabbit serum (control) was bound to 1.5 mg of protein G magnetic Dynabeads (Life Technologies). The coated beads were incubated with 8 mg total protein in TaC12 cell lysates overnight at 4 °C. For the IP of endogenous CIN85 and TaMISHIP in TaC12 and BoMac (control) cells, the anti-CIN85 and anti-TaMISHIP anti sera (300 µl of each polyclonal affinity purified antisera) and IgG from rat serum (12 µg, control) antibodies were added to the lysates (8 mg total protein each) overnight at 4 °C. The next morning 1.5 mg of protein L magnetic Dynabeads were added for 6 hr at 4 °C. After incubation, the beads were washed 3 times with lysis buffer and 3 times with PBS. Proteins were eluted from 30% of the beads in Laemmli SDS-sample buffer and subjected to analysis by Western blot. The remaining 70% of the beads were subjected to on beads tryptic digest and analysis by mass spectrometry on an ESI–QTOF mass spectrometer at the Proteomics and Mass Spectrometry Core Facility, Department of Clinical Research, University of Bern, Switzerland. HEK 293T cells transiently expressing GFP-TaMISHIP fusion proteins were lysed in lysis buffer containing 0.5% NP-40 and subjected to GFP-TRAP co-IP (Chromotek) following the manufacturer’s instructions.

4.7 Sporozoite infection

A Holstein calf was infected by subcutaneous inoculation with 1 × 10\textsuperscript{6} cells infected with T. annulata Ankara 279. The calf was monitored for the progress of infection by taking rectal temperature and examining lymph node biopsy and blood smears for the presence of schizonts and piroplasms, respectively. Unfed nymphal Hyalomma excavatum ticks were applied to the ears of the calf when piroplasms were first detected in the blood smears. After the ticks detached and moulted, the sporozoite stabilitate Ankara 279 was prepared as described by Brown (1987).

Peripheral blood mononuclear cells were isolated from venous bovine blood taken from a jugular vein by flotation on Ficoll-Paque as described previously (Goddeeris & Morrison, 1988). Cells were resuspended in RPMI 1640 medium (containing 2-mM glucose, 5 × 10\textsuperscript{-3}-M 2-mercaptoethanol, 100-IU/ml penicillin, 100-µg/ml streptomycin, and 10% FCS) to obtain 10\textsuperscript{7} cells/ml; 500 µl of cell suspension was mixed with an equal volume of T. annulata Ankara 279 stabilitate cultured in culture medium to obtain an equivalent of one tick/ml. The suspension was incubated for 5 min, 30 min, 1 day, or 3 days at 37 °C with occasional agitation, then centrifuged at 200 g for 5 min and washed twice in PBS. The cells were fixed with 4% PFA in PBS for 10 min at room temperature, washed twice, and stored in PBS containing 0.02% sodium azide. Infections were performed at Adnan Menderes University, Turkey. Ethical statement (SNF), project number PZ00P3_154689 awarded to Kerry Woods. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

AUTHOR CONTRIBUTIONS

S. H. and K. W. performed the conception and design of the study. S. H., K. W., T. C., and D. R. carried out the acquisition, analysis, or interpretation of the data. S. H. and K. W. did the writing of the manuscript.

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**ORCID**

Kerry Woods [http://orcid.org/0000-0003-0357-2613](http://orcid.org/0000-0003-0357-2613)

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SUPPORTING INFORMATION

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