Actin from the apicomplexan *Neospora caninum* (NcACT) has different isoforms in 2D electrophoresis

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Abstract

Apicomplexan parasites have unconventional actins that play a central role in important cellular processes such as apicoplast replication, motility of dense granules, endocytic trafficking and force generation for motility and host cell invasion. In this study, we investigated the actin of the apicomplexan *Neospora caninum* – a parasite associated with infectious abortion and neonatal mortality in livestock. *Neospora caninum* actin was detected and identified in two bands by one-dimensional (1D) western blot and in nine spots by the 2D technique. The mass spectrometry data indicated that *N. caninum* has at least nine different actin isoforms, possibly caused by post-translational modifications. In addition, the C4 pan-actin antibody detected specifically actin in *N. caninum* cellular extract. Extracellular *N. caninum* tachyzoites were treated with toxins that act on actin, jasplakinolide and cytochalasin D. Both substances altered the peripheric cytoplasmic localization of actin on tachyzoites. Our findings add complexity to the study of the apicomplexan actin in cellular processes, since the multiple functions of this important protein might be regulated by mechanisms involving post-translational modifications.

Introduction

Neospora caninum is the causative agent of neosporosis, a disease associated with infectious abortion in livestock and neonatal mortality in beef and milk cattle (Dubey and Schares, 2011). This veterinary pathogen is responsible for significant economic losses estimated to be more than one billion dollars annually worldwide (Reichel *et al.*, 2013).

Actin is an abundant and highly conserved protein ubiquitously found in eukaryotes, with an intrinsic ability to form filaments (*F*-actin) from the organization of monomers (*G*-actin). This protein is responsible for many cellular processes including motility (Pollard and Borisy, 2003; Blanchoin *et al.*, 2014), maintenance of cell structure and polarity (Matsudaira, 1991) and transportation of vesicles and organelles (Kübler and Riezman, 1993; Frölich and Wallach, 2015). A large number of regulation factors are known to modulate actin functions, such as actin-binding proteins (ABPs). The ABPs play important roles in the dynamic regulation of actin (Lappalainen, 2016) as well as small molecules such as ATP and Mg^{+2} (Kang *et al.*, 2013; Kudryashov and Reisler, 2013). Toxins including jasplakinolide (JAS), which provoke actin polymerization (Bubb *et al.*, 2000), and cytochalasin D (CYTD), an actin depolymerizing substance (Cooper, 1987), can regulate actin dynamics. Moreover, actin is also regulated by post-translational modifications (PTMs), i.e. the covalent modifications of protein amino acids. PTMs are responsible for equilibrating the organization and proportion of actin monomers and polymers (Terman and Kashina, 2013).

Organisms from the *Apicomplexa* phylum have unconventional actins (Sahoo *et al.*, 2006; Skillman *et al.*, 2013), similarly to other protozoan such as Amoeba, Trypanosomatids and *Giardia* (Gupta *et al.*, 2015). Apicomplexan parasites comprise veterinary pathogens such as *Neospora*, *Theileria* and *Babesia*, zoonotic agents such as *Toxoplasma gondii* and *Cryptosporidium parvum*, and human pathogens represented by the *Plasmodium* genus. Apicomplexans can infect host cells due to their ability to travel through tissues and penetrate cells using gliding motility (Soldati and Meissner, 2004). *Toxoplasma gondii* and *Plasmodium* spp. have been the central organisms employed for elucidation of apicomplexan motility (Tardieux and Baum, 2016). In the current model of gliding motility, the dynamic association of *F*-actin and myosin A, integrated into the complex of specialized proteins named glideosome, powers the parasite to move forward (Opitz and Soldati, 2002; Baum *et al.*, 2006). Actin has other important roles in mechanisms such as cell replication, motility of dense granules, apicoplasts replication and endocytic trafficking (Smythe *et al.*, 2008; Haase *et al.*, 2015; Heaslip *et al.*, 2016; Periz *et al.*, 2017; Whitelaw *et al.*, 2017).

Here, we investigated *N. caninum* actin (NcACT) using *in silico* and proteomic approaches. NcACT is encoded by one single gene, although different isoforms were identified in one-

dimensional (1D) and 2D SDS-PAGE. Additionally, NcACT was visualized in *N. caninum* tachyzoites, being sensitive to JAS and CYTD effect.

Materials and methods

NcACT sequence selection and in silico analysis

The open-reading frame encoding for NcACT was searched using the keyword 'actin' in ToxoDB 6.0 (Gajria *et al.*, 2008). The selected actin ID was chosen considering the sequence with higher identity with the apicomplexan parasite *T. gondii* actin (TgACT) ACT1 (Dobrowolski *et al.*, 1997), access TGME49_009030. The NcACT primary sequence was aligned with other homologous actin of apicomplexan and non-apicomplexan cells. The alignments were performed using MegAlign (LaserGene, DNASTAR, Madison, USA) and edited by GeneDoc version 2.7.000.

Some PTM sites were predicted in NcACT using computational tools. Ubiquitination prediction was carried out in UbiSite (http://csb.cse.yzu.edu.tw/UbiSite/), *s*-nytrosylation prediction in GPS-SNO 1.0 (Xue *et al.*, 2010) and *K*-acetylation prediction in GPS-PAIL 2.0 (Deng *et al.*, 2016) using high threshold as confidence parameters. The prediction of phosphorylation was performed by NetPhos 3.1 (Blom *et al.*, 1999), sumoylation by SumoPlot Analysis Program (http://www.abgent.com/sumoplot) and *S*-glutathionylation by GSHSite (http://csb.cse.yzu.edu.tw/GSHSite/), considering high confidence level (score ⩾0.75). Carbonylation was predicted by PredCar-Site (Hasan *et al.*, 2017).

Neospora caninum culture

Neospora caninum tachyzoites NC1 (Dubey *et al.*, 1988) were cultivated in Vero cells as described previously (Pereira *et al.*, 2011). The tachyzoites were purified by exclusion chromatography in previously equilibrated PD10 columns (Sephadex G-25, GE Healthcare, Buckinghamshire, UK) in phosphate-buffered saline (PBS).

Protein extraction

Depending on the aim of the experiment, total protein from *N. caninum* or Vero cells was extracted using two different buffers. One method was extraction with the actin-stabilizing buffer (60 mM PIPES, 25 mM HEPES, 10 mM EDTA, 2 mM MgCl$_2$, 125 mM KCl, pH 7.2) containing 10% glycerol, 1% Triton X-100 and 1X protease inhibitor (Complete Mini EDTA free, Roche, Mannheim, Germany) in tachyzoites and Vero cells pellets. The suspension was incubated for 1 h on ice. The total protein extraction was also performed by sonication of the pellet with a sample buffer (7 M urea, 2 M thiourea, 4% CHAPS). The suspensions of both extraction buffers were centrifuged at $9500 \times g$ for 10 min at 4 °C. The supernatants were quantified by the Bradford method (Bradford, 1976) and analysed by SDS-PAGE/Western blot.

One-dimensional and 2D SDS-PAGE

The total protein extracts were resolved by SDS-PAGE in 1D or 2D. For 1D SDS-PAGE, 10 or 12.5% acrylamide gels were run in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) with 120–150 V and stained with Coomassie G-250. For 2D SDS-PAGE, a buffer exchange was necessary, from the actin-stabilizing buffer to a sample buffer (7 M urea, 2 M thiourea, 4% CHAPS) using Microcon concentrator (Millipore, Bedford, USA). Subsequently 5 µL of protease inhibitor (Sigma-Aldrich, St. Louis, USA), 0.2 mM DTT (Thermo Fisher

Scientific, Waltham, USA), 5 µL immobilized pH gradient (IPG) buffer 3–11 NL (GE Healthcare, Uppsala, Sweden) and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, traces of bromophenol blue) were added to 50 µg total protein. The samples were loaded on a 7 cm IPG strip (Rehydrate Immobiline DryStrip gel 3–11 NL, GE) and rehydrated for 18 h at room temperature. The isoelectric focusing (IEF) was conducted in the Ettan IPGPhor 3 (GE Healthcare) at the following conditions: step and hold, 300 V, 0.2 kVh; gradient, 1000 V, 0.3 kVh; gradient, 5000 V, 4 kVh; step and hold, 5000 V, 2.0 kVh (total of 6.5 kVh); at 20 °C. After IEF, the IPG strips were equilibrated for 15 min with DTT 10 mg mL^{-1} in equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS), followed by 25 mg mL^{-1} iodoacetamide incubation in the same conditions. The strips were run in SDS-PAGE 12.5% using 10 mA for 15 min and 20 mA for the rest of the electrophoresis run. The gels were digitalized using LabScan v.5.0 software on a flatbed scanner (Image Scanner, GE).

Western blot

Western blots were performed as described by Pereira *et al.* (2011) with modifications. The SDS-PAGE contents were transferred to polyvinylidene difluoride membranes (0.45 µm Immobilon, EMD Millipore) and the membranes were blocked with PBS-GT [0.8% swine gelatin, Sigma-Aldrich, 0.05% (v/v) Tween-20 in PBS]. Monoclonal antibody anti-β-actin C4 (C4 antibody) (Santa Cruz Biotechnology, Dallas, USA) was used for protein detection and anti-mouse IgG-peroxidase (Sigma-Aldrich) as secondary antibody. Membranes were exposed to radiographic film after being incubated with Luminol (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Rockford, USA) for 5 min. Images were obtained as described for SDS-PAGE.

The anti-β-pan-actin C4 monoclonal (Lessard, 1988) is a broad specificity antibody able to detect actin in monomeric and filamentous forms. Its detected epitope is situated in a highly conserved region of the actin sequence (Fig. 1). Here, a C4 antibody was used to detect NcACT in tachyzoites and Vero cells (control) protein extract.

Mass spectrometry (RP-nanoLC-MS/MS) of actin bands and spots

The bands (1D SDS-PAGE) or spots (2D SDS-PAGE) were excised from acrylamide gels and analysed by nano LC-MS/MS as described (Pollo-Oliveira *et al.*, 2013). The resulting data were searched against *N. caninum* predicted protein database ToxoDB version 28 and Swissprot (The UniProt Consortium, 2017), using Mascot software version 2.6.1 (Matrix Science, London, UK). The search was performed using carbamidomethylation as fixed modification of cysteine and oxidation of methionine as variable modification. The peptide tolerance was set to 50 ppm and 0.6 Da of ion tolerances, allowing for two missed cleavages. Proteins and peptide identifications >95% probability were accepted.

Immunofluorescence

Tachyzoites, purified from 5–7 days old cultures, were divided into four (5×10^6 tachyzoites each) 1.5 mL tubes. The samples were incubated with agitation in a total volume of 500 µL of Roswell Park Memorial Institute (RPMI) 1640 containing 5 µM JAS (Santa Cruz Biotechnology), 2 µM CYTD (Santa Cruz Biotechnology), 1% dimethyl sulfoxide (DMSO) (estimated concentration of this vehicle present in drug incubations) or only
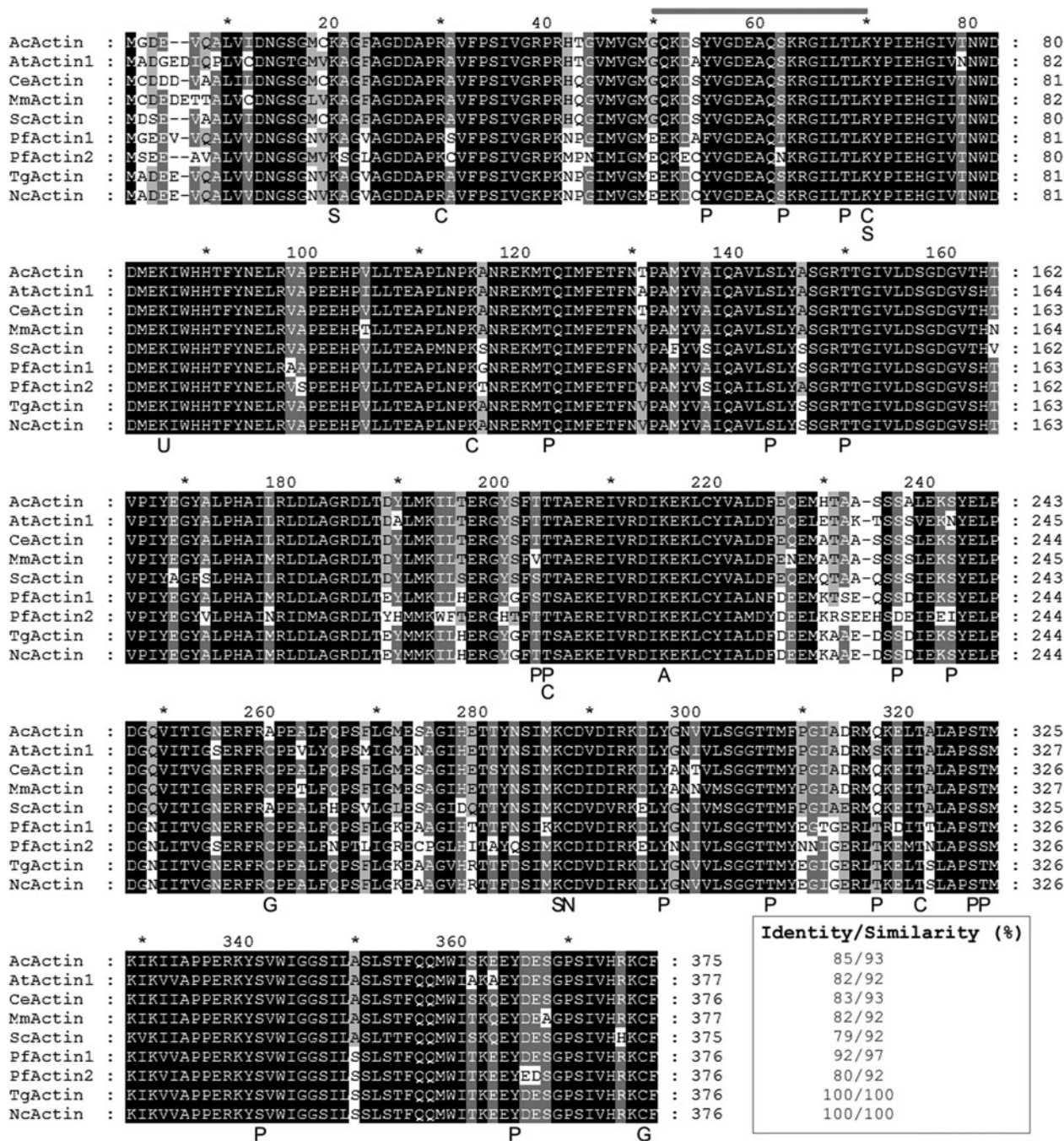
Fig. 1. Multiple alignment of NcACT with representative homologous actins. The sequences are: *Acanthamoeba castellanii* actin (AcActin; NCBI ID CAA23399), *Arabidopsis thaliana* actin 1 (AtActin1; NP850284), *Caenorhabditis elegans* actin (CeActin; CAA34718), *Mus musculus* actin (MmActin; AAA37164), *Saccharomyces cerevisiae* actin (ScActin; P60010), *Plasmodium falciparum* actin 1 (PfActin1; AAA29465), *P. falciparum* actin 2 (PfActin2; AAA29467); *Toxoplasma gondii* (TgActin; XP002369663) and *Neospora caninum* actin (NcActin; ToxoDB ID NCLIV_003440). The primary protein sequences of actin were aligned using Clustal W algorithm. In grey line, the epitope recognized by the C4 antibody. The predicted PTMs are signalized below NcActin sequence. S, sumoylation; C, carbonylation; P, phosphorylation; U, ubiquitinylation; A, k-acetylation; G, s-glutathionylation; N, s-nytrosylation.

RPMI for 15 min at 37 °C. DMSO and RPMI were the negative controls. The tachyzoites were transferred to eight-well chamber slide (Thermo Fisher Scientific, Rochester, USA) and rinsed in PBS, followed by fixation with 3.6% formaldehyde (Sigma-Aldrich) in PBS for 1 h at 25 °C. After rinsing, the tachyzoites were permeabilized with 0.2% Triton X-100 in PBS for 40 min at 25 °C and blocked in PBS with 3% bovine serum albumin (BSA) and 20 mM glycine for 18 h at 10 °C. The samples were rinsed and incubated with monoclonal antibody anti-β-actin C4 (1:500) diluted in PBS containing 0.3% BSA and 2 mM glycine for 45 min at 25 °C, followed by incubation with goat anti-mouse AlexaFluor 488 conjugated antibody (2.5 μL mL^{-1}; Thermo Fisher Scientific, Oregon, USA). The nuclei were stained with 0.5 μM propidium iodide in PBS (Santa Cruz Biotechnology). Images were obtained on a confocal microscope (Leica TCS SP5 Laser Scanning Confocal Microscope, Leica Microsystems, Heidelberg, Germany – Multi-user Laboratory of Confocal Microscopy LMMC-FMRP-USP) using 100× objective in oil immersion, Ar laser at 488 nm for Alexa Fluor 488 and HeNe laser at 543 nm for propidium iodide. The captured images were processed in ImageJ 1.46r (National Institute of Health, USA).
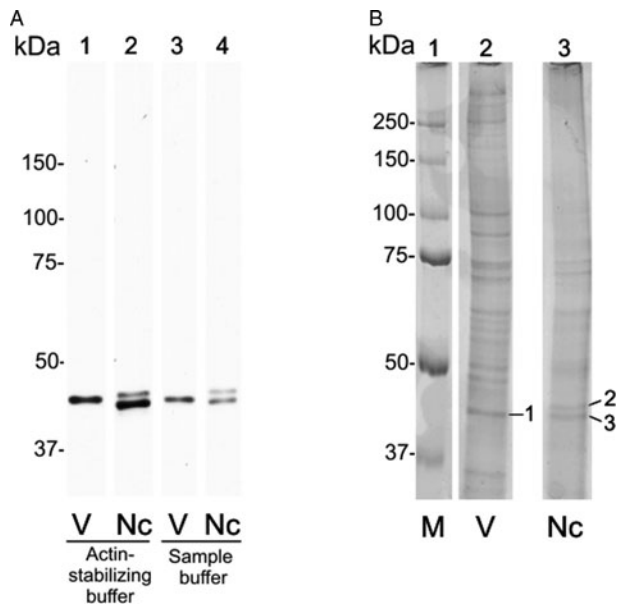
Fig. 2. Detection of NcACT by the C4 antibody and its correspondence in stained gel. A 10% SDS-PAGE was run with *Neospora caninum* or Vero cells extracts until the 25 kDa marker overpassed the gel. Actin was detected by Western blot. (A) Western blot using an anti-actin antibody 1:2000 and anti-mouse antibody 1:8000. *Lanes 1 and 3*: Vero cell total extract in actin-stabilizing buffer and sample buffer, respectively. *Lanes 2 and 4*: *Neospora caninum* total extract in actin-stabilizing buffer and sample buffer, respectively. (B) SDS-PAGE stained with Coomassie G-250. *Lane 1*: marker. Vero cells (*lane 2*) and *N. caninum* (*lane 3*) extracts were obtained in actin-stabilizing buffer. The bands corresponding to anti-actin detection were excised (1–3) and analysed by mass spectrometry. Nc, *N. caninum*; V, Vero cells; M, marker.

Results

NcACT is sequentially identical to TgACT1

Searches on *N. caninum* Liverpool database in ToxoDB using word 'actin' resulted in four identification numbers (ID) annotated as actin: NCLIV_003440 (actin, related), NCLIV_061200 (actin, putative), NCLIV_064780 (actin, putative) and NCLIV_004950 (actin, putative). BLAST searches with the four predicted primary protein sequences indicated that NCLIV_064780 might correspond to actin-related protein 1 (ARP1) homologue, while NCLIV_061200 and NCLIV_004950 might be actin-like proteins 1 (ALP1) and 4 (ALP4) homologues, respectively. Although ARPs share 17–60% identity with conventional actins and are all recognizable as homologous to actin, they are a diverse group with very different functions in cells (Schafer and Schroer, 1999). As previously proposed, ARPs are present in many different organisms, while ALPs are exclusive from the *Apicomplexa* phylum (Gordon and Sibley, 2005; Gordon *et al.*, 2010). The sequence NCLIV_003440 shared actin homology with various species, including *T. gondii* and other apicomplexan parasites. Furthermore, a BLAST search of NCLIV_003440 in *N. caninum* database indicated that *N. caninum* has one single gene copy for actin, like *T. gondii*. This ID, referred to as NcACT, was aligned with multiple homologous actins from representative and apicomplexan organisms and showed 100% identity with TgACT, followed by 92% identity with *Plasmodium falciparum* actin 1, and 85% identity with *Acanthamoeba castellanii* actin (Fig. 1).

C4 antibody detected two bands in 1D SDS-PAGE

On 12.5% SDS-PAGE, the C4 antibody detected a single ~43 kDa band in both extracts of *N. caninum* and Vero cells (unpublished results). When *N. caninum* total extracts was run on a 10% SDS-PAGE subjected to a longer run, the single band was

interestingly divided in two bands of ~42 and ~44 kDa (Fig. 2A, wells 2 and 4). In the control, a single ~43 kDa band in Vero cells remained (Fig. 2A, wells 1 and 3). The same result was achieved in two different conditions: using Vero cells extract supernatants in actin-stabilizing buffer (Fig. 2A, wells 1 and 2) or in a denaturing buffer (Fig. 2A, wells 3 and 4).

The SDS-PAGE bands containing *N. caninum* and Vero cells extracts were submitted to nanoLC-MS/MS (numbered as indicated in Fig. 2B). The three bands exhibited hits for actin (Table 1, ranked per the probability score). In ToxoDB, the ID NCLIV_003440 (NcACT) was identified in the two bands (2 and 3) that contained *N. caninum* extract. The same ID for NcACT at the Vero cells extract band 1 was obtained due to the presence of conserved peptides (Table 1). The search at Swissprot identified vertebrate cytoplasmic actins in the Vero cells and *N. caninum* bands (1 and 3, respectively) but also in TgACT (band 2, Table 1). The cross IDs between both extracts was expected, not only because of Vero cells debris that may have remained after tachyzoite purification, but also due to a high sequence identity between NcACT and Vero cell β-actin (83%, unpublished results). The significant and top-ranked IDs showed that all peptides from the ToxoDB searches of Vero cells band matched the Vero cell β-actin sequence (Supplementary Material 5). On the other hand, peptides from band 3 did not match Vero cell β-actin, apart from one present at NcACT (bold peptide, Supplementary Material 5; Table 1). In addition, the mass spectrometry allowed the identification of several other proteins, despite the presence of actin in every analysis (Supplementary Material 1–4). Overall, the MS results showed that bands 1 and 3 are composed of vertebrate actin, and band 2 is composed of NcACT. However, the evidence of NcACT was found in band 3, indicating the presence of isoforms.

NcACT has different isoforms in 2D SDS-PAGE

A double row was observed at the *N. caninum* extract 2D Western blot (like the two bands at the 1D Western blot). The spots were numbered 1–9 (Fig. 3A) in comparison to the control Vero cells extract with three spots (pI 5.2–5.7) (Fig. 3D). Nine spots (with pI 5.2–5.8) were detected by the C4 antibody in *N. caninum* extract, co-localized at a Coomassie-stained SDS-PAGE and submitted to nanoLC-MS/MS (Fig. 3A and B).

The three Vero cells spots presented hits for actin in Swissprot and ToxoDB (Table 2). Nevertheless, all significant and top-ranked peptides from ToxoDB searches were identical to both NcACT and Vero cell β-actin respective sequence regions (Supplementary Material 6). Spots from *N. caninum* extract were also identified as actin in both databases (Table 3) but in contrast to the Vero cells spots, presented at least one NcACT exclusive peptide when compared with Vero cell β-actin at ToxoDB searches (Supplementary Material 7). Except for spot 1, all *N. caninum* spots presented hits for TgACT in Swissprot searches (Supplementary Material 3). Additionally, the higher scores of protein accessions in Swissprot for *N. caninum* spots 3, 4, 5 and 6 were hits for TgACT (Table 3). Although *N. caninum* spot 1 did not have hits for TgACT in Swissprot searches, the peptide identifications to NcACT in ToxoDB matched exclusively with NcACT sequence when compared with Vero cell β-actin (Supplementary Material 7).

Collectively, these results provided evidence that the C4 antibody is able to detect actin in *N. caninum* extract and that NcACT presents nine different isoforms, varying in molecular weight and isoelectric points between them.

PTMs are computationally predicted in NcACT

The analysis of NcACT by computational tools revealed possible target sites for PTMs. One site was predicted for ubiquitination,

Table 1. Actin hits from mass spectrometry analysis of *Neospora caninum* and Vero cells extract. Western blot bands detected by the C4 antibody were localized with their correspondent bands at 1D SDS-PAGE for excision and analysis by MS/MS. Hits with the higher scores for actin identification in Swissprot and ToxoDB are shown

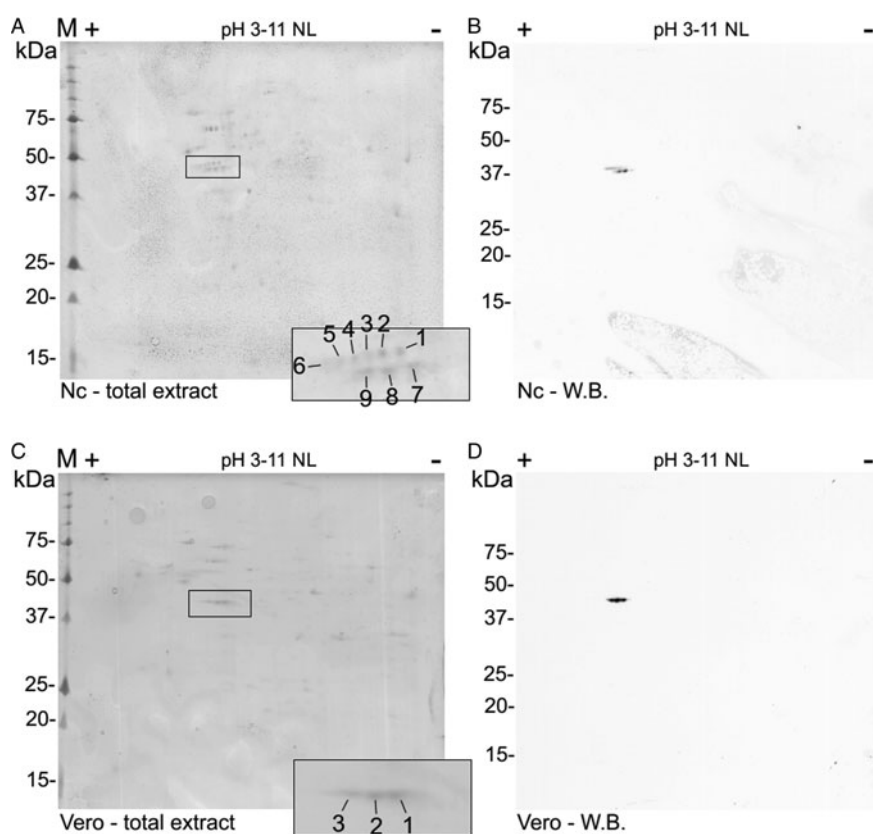| Cell extract	Band	Swissprot ID	Swissprot score	Coverage (%)	ToxoDB ID	ToxoDB score	Coverage (%)
Vero	1	P53478 Actin, cytoplasmic type 5 (*Gallus gallus*)	2186	70	NCLIV_003440	333	24
N. caninum	2	P53476 Actin (*Toxoplasma gondii*)	518	58	NCLIV_003440	943	60
	3	P53505 Actin, cytoplasmic type 5 (*Xenopus laevis*)	1566	68	NCLIV_003440	170	20

Fig. 3. Two-dimensional detection of NcACT. Two-dimensional SDS-PAGE was performed using gel strips with non-linear pH range 3–11 previously focused with *Neospora caninum* (A and B) or Vero cell (C and D) total extracts. The strips were run in 12.5% SDS-PAGE. (A) and (C) Coomassie-G250 stained gels containing *N. caninum* and Vero cells extracts, respectively. The correspondent spots detected by Western blot were numbered and highlighted inside black rectangles. (B) and (D) Western blot from SDS-PAGE containing *N. caninum* and Vero cell extracts, respectively. The actin isoforms were detected with anti-actin antibody 1:2000 and anti-mouse antibody 1:8000. W.B., Western blot.

s-nitrosylation and *K*-acetylation, 17 sites for phosphorylation, two sites for *s*-glutathionylation, five sites for carbonylation and three sites for sumoylation (Fig. 1 and Supplementary Material 8).

NcACT is located peripherally on the cytoplasm

The tachyzoites incubated with RPMI presented a well-defined peripheral location of NcACT in the cytoplasm (Fig. 4A). A similar pattern was observed when control tachyzoites were incubated with 1% DMSO (Fig. 4B). However, in tachyzoites incubated with JAS, NcACT was found concentrated in localized dots. The actin dots were located predominantly in apical and/or posterior regions (Fig. 4C). CYTD-treated tachyzoites presented a weak diffuse cytoplasmic staining of NcACT (Fig. 4D).

Discussion

The characterization of actin from the apicomplexan *N. caninum* (NcACT) was performed by computational analysis, in-gel identification and localization in tachyzoites treated with toxins that affect actin. The mechanisms of actin regulation have been extensively studied as this protein has great importance for cellular functions. Apicomplexan organisms have one of the most divergent and primitive actin (Vahokoski *et al.*, 2014), known for their unconventional characteristics (Dobrowolski and Sibley, 1996; Wetzel *et al.*, 2003). Consequently, it is difficult to conciliate *in silico* observations of TgACT isodesmic polymerization (Skillman *et al.*, 2013) with the recent *in vivo* observation of critical concentration during filaments formation (Periz *et al.*, 2017). Additionally, *Plasmodium* actin 1 (PfACT1) was reported to exhibit a polymerization-dependent nucleation (Kumpula *et al.*, 2017). We demonstrated that NcACT is composed of different isoforms. The computational analysis showed NcACT as sequentially identical to TgACT, thus they may present similar *in vitro* polymerization characteristics. In contrast, the detection of actin by the C4 antibody showed significant differences. While actin from *T. gondii*, *Plasmodium berghei* and *P. falciparum* are detected by the C4 antibody as a single predicted actin band in 1D Western blot (Dobrowolski *et al.*, 1997; Angrisano *et al.*, 2012b), NcACT is detected by the same antibody as two distinct

Table 2. Actin hits from mass spectrometry analysis of Vero cells extract. Western blot-correspondent spots detected by the C4 antibody were localized in their correspondent spots at 2D SDS-PAGE for excision and analysis by MS/MS. Hits with the higher scores for actin identification in Swissprot and ToxoDB are shown

Cell extract	Spot	NCBI ID	NCBI score	Coverage (%)	ToxoDB ID	ToxoDB score	Coverage (%)
Vero	1	P53505 Actin, cytoplasmic type 5 (*Xenopus laevis*)	1566	68	NCLIV_003440	170	20
	2	P79818 Actin, cytoplasmic (*Oryzias latipes*)	1342	61	NCLIV_003440	174	33
	3	P18600 Actin (*Artemia* sp.)	937	45	NCLIV_003440	141	18

Table 3. Actin hits from mass spectrometry analysis of *Neospora caninum* extract. Western blot spots detected by C4 antibody were localized with their correspondent spots at 2D SDS-PAGE for excision and analysis by MS/MS. Hits with the higher scores for actin identification in Swissprot and ToxoDB are shown

Cell extract	Spot	Swissprot ID	Swissprot score	Coverage (%)	ToxoDB ID	ToxoDB score	Coverage (%)
N. caninum	1	P60712 Actin cytoplasmic 1 (*Bos taurus*)	66	22	NCLIV_003440	33	10
	2	P68556 Actin (*Diphyllobothrium dendriticum*)	91	38	NCLIV_003440	97	31
	3	P53476 Actin (*Toxoplasma gondii*)	256	61	NCLIV_003440	496	63
	4	P53476 Actin (*T. gondii*)	361	65	NCLIV_003440	698	65
	5	P53476 Actin (*T. gondii*)	376	64	NCLIV_003440	724	66
	6	P53476 Actin (*T. gondii*)	66	31	NCLIV_003440	96	32
	7	Q5JAK2 Actin (*Rana lessonae*)	821	50	NCLIV_003440	118	31
	8	P53505 Actin, cytoplasmic type 5 (*Xenopus laevis*)	1081	57	NCLIV_003440	266	48
	9	P79818 Actin cytoplasmic 1 (*Oryzias latipes*)	973	46	NCLIV_003440	213	39

bands with different lysis conditions. This is a unique observation for an apicomplexan actin so far. The possibility of a non-specific reaction or exclusive detection of Vero cells actin in *N. caninum* extract by the C4 antibody was excluded, as both bands were identified as NcACT proteins by MS/MS. Moreover, no hits for ALPs or ARPs were obtained within the analysis. However, hits for the ALP 1 and ARP 1 (NCLIV_061200 and NCLIV_ 064780, respectively), identified here in BLAST searches, were previously identified by a quantitative approach in *N. caninum* secretome (Pollo-Oliveira *et al.*, 2013). The same approach also detected other members of an ALP family (Pollo-Oliveira *et al.*, 2013).

Searches in the *N. caninum* genome at the ToxoDB database confirmed that NcACT is expressed by a single-copy gene. These observations indicated that the nine different isoforms described represent PTMs. In multicellular organisms, the actin isoforms are encoded by separate genes. In mammals, the variation of *N*-terminal residues and intracellular localization of the six isoforms may determine their function (Perrin and Ervasti, 2010). Plants have a variable number of actin family genes that may be more diversified in plant organisms with complex tissues (Slajcherová *et al.*, 2012). Additionally, the actins of the apicomplexan *Plasmodium* spp are encoded by two genes (I and II),

being actin I expressed throughout the parasite life cycle, and actin II expressed by the reproductive forms (Wesseling *et al.*, 1989; Deligianni *et al.*, 2011; Andreadaki *et al.*, 2014). Nevertheless, TgACT is encoded by one gene (Dobrowolski *et al.*, 1997), and the presence of multiple isoforms were detected in *T. gondii* global proteome analysis, even though specific actin findings were not discussed in the study (Xia *et al.*, 2008). Additionally, actin was identified in two spots of approximately pI 5 in a general 2D electrophoresis map of *N. caninum* (Lee *et al.*, 2003). Multiple actin isoforms with stage-specific pattern were detected in *Trypanosoma cruzi* extracts in 2D electrophoresis by anti-*T. cruzi* actin antibody (Cevallos *et al.*, 2011). The 2D SDS-PAGE is an appropriate approach to detect covalent modifications in side chains of proteins, i.e. PTMs, carried out either during or after translation. The PTMs of actin are important regulatory mechanisms to the function of actin in a cell (Terman and Kashina, 2013). A known PTM of actin is acetylation, performed by aminopeptidases that remove the *N*-terminal methionine and modify the immediate subsequent amino acids (Rubenstein and Martin, 1983). The ubiquitination of actin (arthrin) has been reported in arthropods (Bullard *et al.*, 1985; Burgess *et al.*, 2004) and in PfACT1 (Field *et al.*, 1993; González-López *et al.*,
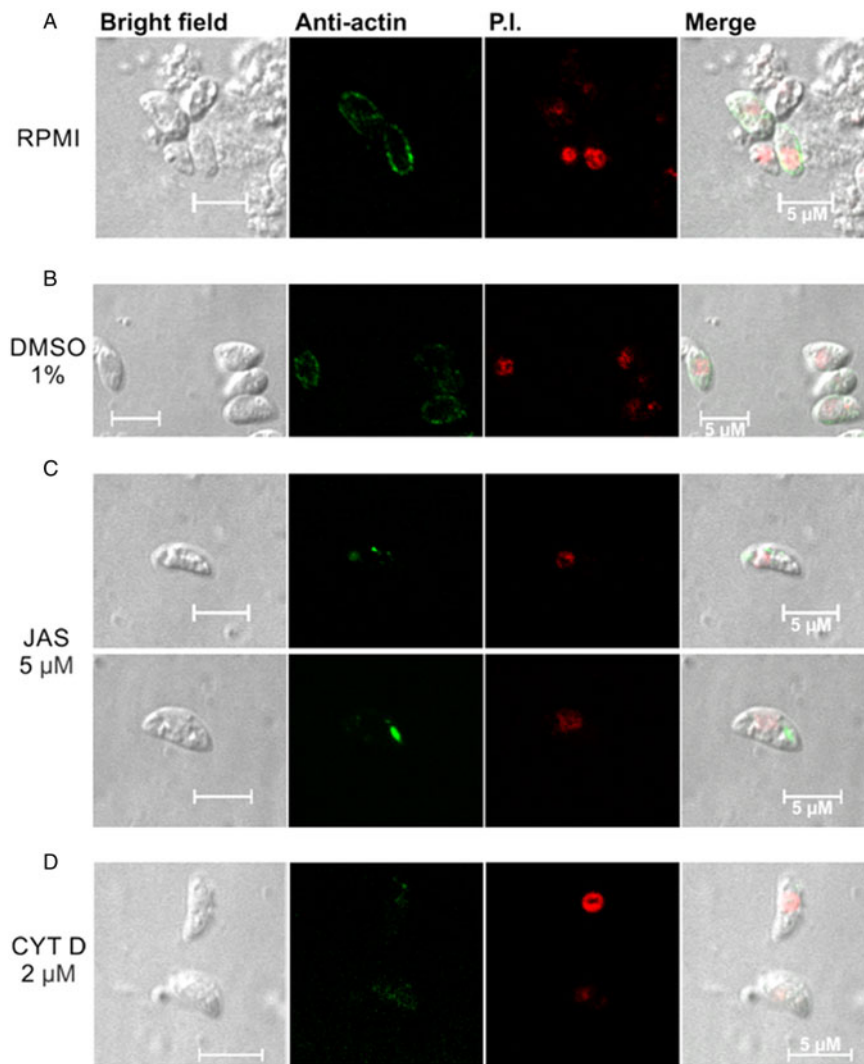
Fig. 4. Immunolocalization of NcACT from *Neospora caninum* tachyzoites. Tachyzoites were treated for 15 min at 37 °C with drugs that affect the cytoskeleton. The C4 antibody (1:500) was used to detect NcACT. The detection was visualized with the anti-mouse Alexa Flour 488 conjugated antibody and the nucleus was marked with propidium iodide (P.I.). (A) The tachyzoites were incubated with only RPMI. (B) Incubation with 1% DMSO in RPMI. (C) Incubation with 5 μM jasplakinolide (JAS). (C) Incubation with 2 μM cytochalasin D (CYTD). The white bars indicate 5 μm.

2017). Other PTMs such as arginylation (Karakozova *et al.*, 2006), phosphorylation (Kishi *et al.*, 1998; Gu *et al.*, 2003), carbonylation (Castro *et al.*, 2012), *s*-nitrosylation (Lu *et al.*, 2009), methylation (Nyman *et al.*, 2002), glutathionylation (Sakai *et al.*, 2012) and sumoylation (Alonso *et al.*, 2015) were characterized in actin. Among the previously characterized actin PTMs, the study computationally predicted sites of ubiquitination, phosphorylation, carbonylation, *s*-nitrosylation, glutathionylation, *K*-acetylation and sumoylation in NcACT. The present data indicated that NcACT may have its function regulated by mechanisms other than ABPs.

The distribution of actin in *N. caninum* tachyzoites was different from *T. gondii* and *Plasmodium*. Dobrowolski *et al.* (1997) observed, in extracellular *T. gondii* marked with the C4 antibody, a more intense peripheral localization and a cytoplasmic diffuse signal. *Plasmodium berghei* sporozoites also had a peripheral concentration of actin, although a more uniform fluorescence was localized throughout the cytoplasm in merozoites and ookinetes (Angrisano *et al.*, 2012b). Fixed *P. berghei* ookinetes presented actin concentrated at the extreme apical pole (Siden-Kiamos *et al.*, 2006). The peripheral presence of NcACT is coincident with the localization of the glideosome between the inner membrane complex and the plasmatic membrane of the tachyzoite (Heintzelman, 2015). JAS and CYTD are valuable tools that have been extensively used and have enabled useful insights into the characterization of actin from *Apicomplexa* (Dobrowolski *et al.*, 1997; Wetzel *et al.*, 2003; Angrisano *et al.*, 2012b; Drewry and Sibley, 2015; Whitelaw *et al.*, 2017). The current study demonstrated that NcACT is sensitive to these toxins since the JAS and CYTD treatments modified the localization of actin in the tachyzoites. The dots of actin localized in the cytoplasm after incubation with JAS, specifically in the apical and/or posterior regions, most likely represent the induction of the polymerization. A similar pattern was observed in fixed *P. berghei* ookinetes incubated with a monoclonal anti-actin antibody (Siden-Kiamos *et al.*, 2006) or expressing constitutive GFP-Pb actin (Angrisano *et al.*, 2012a). The apical protrusions observed in JAS-treated *T. gondii* tachyzoites (Shaw and Tilney, 1999; Wetzel *et al.*, 2003; Angrisano *et al.*, 2012a) and *P. falciparum* merozoites (Mizuno *et al.*, 2002) were not detected in our experimental conditions. The *N. caninum* tachyzoites treated with CYTD presented a weak and diffuse signal for actin, indicating actin filaments disruption. Intracellular *T. gondii* tachyzoites had the actin networking collapsed after CYTD treatment (Periz *et al.*, 2017), whereas in *P. falciparum*, the localization of actin remained similar to the control when treated with the same substance (Smythe *et al.*, 2008).

In summary, our findings demonstrated that the actin localization on *N. caninum* tachyzoites and detection in Western blot differed from the findings previously described for the related coccidian *T. gondii*. In addition, the data strongly suggest that post-translational processing occurs in NcACT. The PTMs add complexity to the analysis of important cellular functions such as those determined by the actin dynamics. The study of PTMs in apicomplexan parasites may explain the specificities of their

unconventional actin dynamics and complement the information of the functional singularities caused by divergences in amino acids sequences between species.

References

Alonso A, Greenlee M, Matts J, Kline J, Davis KJ and Miller RK (2015) Emerging roles of sumoylation in the regulation of actin, microtubules, intermediate filaments, and septins. *Cytoskeleton (Hoboken)* **72**, 305–339.

Andreadaki M, Morgan RN, Deligianni E, Kooij TW, Santos JM, Spanos L, Matuschewski K, Louis C, Mair GR and Siden-Kiamos I (2014) Genetic crosses and complementation reveal essential functions for the *Plasmodium* stage-specific actin2 in sporogonic development. *Cell Microbiology* **16**, 751–767.

Angrisano F, Delves MJ, Sturm A, Mollard V, McFadden GI, Sinden RE and Baum J (2012*a*) A GFP-actin reporter line to explore microfilament dynamics across the malaria parasite lifecycle. *Molecular and Biochemical Parasitology* **182**, 93–96.

Angrisano F, Riglar DT, Sturm A, Volz JC, Delves MJ, Zuccala ES, Turnbull L, Dekiwadia C, Olshina MA, Marapana DS, Wong W, Mollard V, Bradin CH, Tonkin CJ, Gunning PW, Ralph SA, Whitchurch CB, Sinden RE, Cowman AF, McFadden GI and Baum J (2012*b*) Spatial localisation of actin filaments across developmental stages of the malaria parasite. *PLoS ONE* **7**, e32188.

Baum J, Papenfuss AT, Baum B, Speed TP and Cowman AF (2006) Regulation of apicomplexan actin-based motility. *Nature Reviews Microbiology* **4**, 621–628.

Blanchoin L, Boujemaa-Paterski R, Sykes C and Plastino J (2014) Actin dynamics, architecture, and mechanics in cell motility. *Physiological Reviews* **94**, 235–263.

Blom N, Gammeltoft S and Brunak S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology* **294**, 1351–1362.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Bubb M, Spector I, Beyer B and Fosen K (2000) Effects of jasplakinolide on the kinetics of actin polymerization – an explanation for certain in vivo observations. *Journal of Biological Chemistry* **275**, 5163–5170.

Bullard B, Bell J, Craig R and Leonard K (1985) Arthrin: a new actin-like protein in insect flight muscle. *Journal of Molecular Biology* **182**, 443–454.

Burgess S, Walker M, Knight P, Sparrow J, Schmitz S, Offer G, Bullard B, Leonard K, Holt J and Trinick J (2004) Structural studies of arthrin: monoubiquitinated actin. *Journal of Molecular Biology* **341**, 1161–1173.

Castro J, Ott C, Jung T, Grune T and Almeida H (2012) Carbonylation of the cytoskeletal protein actin leads to aggregate formation. *Free Radical Biology and Medicine* **53**, 916–925.

Cevallos AM, Segura-Kato YX, Merchant-Larios H, Manning-Cela R, Alberto Hernández-Osorio L, Márquez-Dueñas C, Ambrosio JR, Reynoso-Ducoing O and Hernández R (2011) *Trypanosoma cruzi*: multiple actin isovariants are observed along different developmental stages. *Experimental Parasitology* **127**, 249–259.

Cooper JA (1987) Effects of cytochalasin and phalloidin on actin. *Journal of Cell Biology* **105**, 1473–1478.

Deligianni E, Morgan RN, Bertuccini L, Kooij TW, Laforge A, Nahar C, Poulakakis N, Schüler H, Louis C, Matuschewski K and Siden-Kiamos I (2011) Critical role for a stage-specific actin in male exflagellation of the malaria parasite. *Cell Microbiology* **13**, 1714–1730.

Deng W, Wang C, Zhang Y, Xu Y, Zhang S, Liu Z and Xue Y (2016) GPS-PAIL: prediction of lysine acetyltransferase-specific modification sites from protein sequences. *Scientific Reports* **6**, 39787.

Dobrowolski JM and Sibley LD (1996) Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84**, 933–939.

Dobrowolski J, Niesman I and Sibley L (1997) Actin in the parasite *Toxoplasma gondii* is encoded by a single copy gene, ACT1 and exists primarily in a globular form. *Cell Motility and the Cytoskeleton* **37**, 253–262.

Drewry LL and Sibley LD (2015) Toxoplasma actin is required for efficient host cell invasion. *MBio* **6**, e00557.

Dubey J and Schares G (2011) Neosporosis in animals – the last five years. *Veterinary Parasitology* **180**, 90–108.

Dubey J, Hattel A, Lindsay D and Topper M (1988) Neonatal *Neospora caninum* infection in dogs – isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medical Association* **193**, 1259–1263.

Field SJ, Pinder JC, Clough B, Dluzewski AR, Wilson RJ and Gratzer WB (1993) Actin in the merozoite of the malaria parasite, *Plasmodium falciparum*. *Cell Motility Cytoskeleton* **25**, 43–48.

Frölich S and Wallach M (2015) F-actin distribution and function during sexual development in *Eimeria maxima*. *Parasitology* **142**, 855–864.

Gajria B, Bahl A, Brestelli J, Dommer J, Fischer S, Gao X, Heiges M, Iodice J, Kissinger JC, Mackey AJ, Pinney DF, Roos DS, Stoeckert CJ, Wang H and Brunk BP (2008) ToxoDB: an integrated *Toxoplasma gondii* database resource. *Nucleic Acids Research* **36**, D553–D556.

González-López L, Carballar-Lejarazú R, Arrevillaga Boni G, Cortés-Martínez L, Cázares-Raga FE, Trujillo-Ocampo A, Rodríguez MH, James AA and Hernández-Hernández FC (2017) Lys48 ubiquitination during the intraerythrocytic cycle of the rodent malaria parasite, *Plasmodium chabaudi*. *PLoS ONE* **12**, e0176533.

Gordon JL and Sibley LD (2005) Comparative genome analysis reveals a conserved family of actin-like proteins in apicomplexan parasites. *BMC Genomics* **6**, 179.

Gordon JL, Buguliskis JS, Buske PJ and Sibley LD (2010) Actin-like protein 1 (ALP1) is a component of dynamic, high molecular weight complexes in *Toxoplasma gondii*. *Cytoskeleton (Hoboken)* **67**, 23–31.

Gu L, Zhang H, Chen Q and Chen J (2003) Calyculin A-induced actin phosphorylation and depolymerization in renal epithelial cells. *Cell Motility and the Cytoskeleton* **54**, 286–295.

Gupta CM, Thiyagarajan S and Sahasrabuddhe AA (2015) Unconventional actins and actin-binding proteins in human protozoan parasites. *International Journal of Parasitology* **45**, 435–447.

Haase S, Zimmermann D, Olshina MA, Wilkinson M, Fisher F, Tan YH, Stewart RJ, Tonkin CJ, Wong W, Kovar DR and Baum J (2015) Disassembly activity of actin-depolymerizing factor (ADF) is associated with distinct cellular processes in apicomplexan parasites. *Molecular Biology of the Cell* **26**, 3001–3012.

Hasan MA, Li J, Ahmad S and Molla MK (2017) PredCar-site: carbonylation sites prediction in proteins using support vector machine with resolving data imbalanced issue. *Analytical Biochemistry* **525**, 107–113.

Heaslip AT, Nelson SR and Warshaw DM (2016) Dense granule trafficking in *Toxoplasma gondii* requires a unique class 27 myosin and actin filaments. *Molecular Biology of the Cell* **27**, 2080–2089.

Heintzelman MB (2015) Gliding motility in apicomplexan parasites. *Seminars in Cell & Developmental Biology* **46**, 135–142.

Kang H, Bradley MJ, Elam WA and De La Cruz EM (2013) Regulation of actin by ion-linked equilibria. *Biophysical Journal* **105**, 2621–2628.

Karakozova M, Kozak M, Wong C, Bailey A, Yates J, Mogilner A, Zebroski H and Kashina A (2006) Arginylation of beta-actin regulates actin cytoskeleton and cell motility. *Science* **313**, 192–196.

Kishi Y, Clements C, Mahadeo D, Cotter D and Sameshima M (1998) High levels of actin tyrosine phosphorylation: correlation with the dormant state of dictyostelium spores. *Journal of Cell Science* **111**, 2923–2932.

Kübler E and Riezman H (1993) Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO Journal* **12**, 2855–2862.

Kudryashov DS and Reisler E (2013) ATP and ADP actin states. *Biopolymers* **99**, 245–256.

Kumpula EP, Pires I, Lasiwa D, Piirainen H, Bergmann U, Vahokoski J and Kursula I (2017) Apicomplexan actin polymerization depends on nucleation. *Scientific Reports* **7**, 12137.

Lappalainen P (2016) Actin-binding proteins: the long road to understanding the dynamic landscape of cellular actin networks. *Molecular Biology of the Cell* **27**, 2519–2522.

Lee E, Kim J, Shin Y, Shin G, Suh M, Kim D, Kim Y, Kim G and Jung T (2003) Establishment of a two-dimensional electrophoresis map for *Neospora caninum* tachyzoites by proteomics. *Proteomics* **3**, 2339–2350.

Lessard JL (1988) Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motility and the Cytoskeleton* **10**, 349–362.

Lu J, Katano T, Okuda-Ashitaka E, Oishi Y, Urade Y and Ito S (2009) Involvement of S-nitrosylation of actin in inhibition of neurotransmitter release by nitric oxide. *Molecular Pain* **5**, 58.

Matsudaira P (1991) Modular organization of actin crosslinking proteins. *Trends in Biochemical Sciences* **16**, 87–92.

Mizuno Y, Makioka A, Kawazu S, Kano S, Kawai S, Akaki M, Aikawa M and Ohtomo H (2002) Effect of jasplakinolide on the growth, invasion, and actin cytoskeleton of *Plasmodium falciparum*. *Parasitology Research* **88**, 844–848.

Nyman T, Schuler H, Korenbaum E, Schutt C, Karlsson R and Lindberg U (2002) The role of MeH73 in actin polymerization and ATP hydrolysis. *Journal of Molecular Biology* **317**, 577–589.

Opitz C and Soldati D (2002) 'The glideosome': a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*. *Molecular Microbiology* **45**, 597–604.

Pereira LM, Candido-Silva JA, De Vries E and Yatsuda AP (2011) A new thrombospondin-related anonymous protein homologue in *Neospora caninum* (NcMIC2-like1). *Parasitology* **138**, 287–297.

Periz J, Whitelaw J, Harding C, Gras S, Del Rosario Minina MI, Latorre-Barragan F, Lemgruber L, Reimer MA, Insall R, Heaslip A and Meissner M (2017) *Toxoplasma gondii* F-actin forms an extensive filamentous network required for material exchange and parasite maturation. *Elife* **6**, e24119.

Perrin BJ and Ervasti JM (2010) The actin gene family: function follows isoform. *Cytoskeleton (Hoboken)* **67**, 630–634.

Pollard T and Borisy G (2003) Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112**, 453–465.

Pollo-Oliveira L, Post H, Acencio ML, Lemke N, van den Toorn H, Tragante V, Heck AJ, Altelaar AF and Yatsuda AP (2013) Unravelling the *Neospora caninum* secretome through the secreted fraction (ESA) and quantification of the discharged tachyzoite using high-resolution mass spectrometry-based proteomics. *Parasites & Vectors* **6**, 335.

Reichel M, Ayanegui-Alcerreca M, Gondim L and Ellis J (2013) What is the global economic impact of *Neospora caninum* in cattle – The billion dollar question. *International Journal for Parasitology* **43**, 133–142.

Rubenstein PA and Martin DJ (1983) NH2-terminal processing of *Drosophila melanogaster* actin. Sequential removal of two amino acids. *Journal of Biological Chemistry* **258**, 11354–11360.

Sahoo N, Beatty W, Heuser J, Sept D and Sibley L (2006) Unusual kinetic and structural properties control rapid assembly and turnover of actin in the parasite *Toxoplasma gondii*. *Molecular Biology of the Cell* **17**, 895–906.

Sakai J, Li J, Subramanian KK, Mondal S, Bajrami B, Hattori H, Jia Y, Dickinson BC, Zhong J, Ye K, Chang CJ, Ho YS, Zhou J and Luo HR (2012) Reactive oxygen species-induced actin glutathionylation controls actin dynamics in neutrophils. *Immunity* **37**, 1037–1049.

Schafer DA and Schroer TA (1999) Actin-related proteins. *Annual Review of Cell Developmental Biology* **15**, 341–363.

Shaw M and Tilney L (1999) Induction of an acrosomal process in *Toxoplasma gondii*: visualization of actin filaments in a protozoan parasite. *Proceedings of the National Academy of Sciences of the USA* **96**, 9095–9099.

Siden-Kiamos I, Pinder JC and Louis C (2006) Involvement of actin and myosins in *Plasmodium berghei* ookinete motility. *Molecular and Biochemical Parasitology* **150**, 308–317.

Skillman KM, Ma CI, Fremont DH, Diraviyam K, Cooper JA, Sept D and Sibley LD (2013) The unusual dynamics of parasite actin result from isodesmic polymerization. *Nature Communications* **4**, 2285.

Slajcherová K, Fišerová J, Fischer L and Schwarzerová K (2012) Multiple actin isotypes in plants: diverse genes for diverse roles? *Frontiers in Plant Science* **3**, 226.

Smythe WA, Joiner KA and Hoppe HC (2008) Actin is required for endocytic trafficking in the malaria parasite *Plasmodium falciparum*. *Cellular Microbiology* **10**, 452–464.

Soldati D and Meissner M (2004) Toxoplasma as a novel system for motility. *Current Opinion in Cell Biology* **16**, 32–40.

Tardieux I and Baum J (2016) Reassessing the mechanics of parasite motility and host-cell invasion. *The Journal of Cell Biology* **214**, 507–515.

Terman JR and Kashina A (2013) Post-translational modification and regulation of actin. *Current Opinion in Cell Biology* **25**, 30–38.

The UniProt Consortium (2017) Uniprot: the universal protein knowledgebase. *Nucleic Acids Research* **45**, D158–D169.

Vahokoski J, Bhargav SP, Desfosses A, Andreadaki M, Kumpula EP, Martinez SM, Ignatev A, Lepper S, Frischknecht F, Sidén-Kiamos I, Sachse C and Kursula I (2014) Structural differences explain diverse functions of *Plasmodium* actins. *PLoS Pathogens* **10**, e1004091.

Wesseling JG, Snijders PJ, van Someren P, Jansen J, Smits MA and Schoenmakers JG (1989) Stage-specific expression and genomic organization of the actin genes of the malaria parasite *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **35**, 167–176.

Wetzel DM, Håkansson S, Hu K, Roos D and Sibley LD (2003) Actin filament polymerization regulates gliding motility by apicomplexan parasites. *Molecular Biology of the Cell* **14**, 396–406.

Whitelaw JA, Latorre-Barragan F, Gras S, Pall GS, Leung JM, Heaslip A, Egarter S, Andenmatten N, Nelson SR, Warshaw DM, Ward GE and Meissner M (2017) Surface attachment, promoted by the actomyosin system of *Toxoplasma gondii* is important for efficient gliding motility and invasion. *BMC Biology* **15**, 1.

Xia D, Sanderson SJ, Jones AR, Prieto JH, Yates JR, Bromley E, Tomley FM, Lal K, Sinden RE, Brunk BP, Roos DS and Wastling JM (2008) The proteome of *Toxoplasma gondii*: integration with the genome provides novel insights into gene expression and annotation. *Genome Biology* **9**, R116.

Xue Y, Liu Z, Gao X, Jin C, Wen L, Yao X and Ren J (2010) GPS-SNO: computational prediction of protein S-nitrosylation sites with a modified GPS algorithm. *PLoS ONE* **5**, e11290.