

F-actin distribution and function during sexual development in *Eimeria maxima*

SONJA FRÖLICH* and MICHAEL WALLACH

The iThree Institute, School of Medical and Molecular Biosciences, University of Technology Sydney, PO Box 123, Broadway, Sydney, New South Wales 2007, Australia

(Received 15 November 2014; revised 13 January 2015; accepted 1 February 2015; first published online 24 March 2015)

SUMMARY

To determine the involvement of the actin cytoskeleton in macrogametocyte growth and oocyst wall formation, freshly purified macrogametocytes and oocysts were stained with Oregon Green 514 conjugated phalloidin to visualize F-actin microfilaments, while Evans blue staining was used to detect type 1 wall forming bodies (WFB1s) and the outer oocyst wall. The double-labelled parasites were then analysed at various stages of sexual development using three-dimensional confocal microscopy. The results showed F-actin filaments were distributed throughout the entire cytoplasm of mature *Eimeria maxima* macrogametocytes forming a web-like meshwork of actin filaments linking the type 1 WFBs together into structures resembling ‘beads on a string’. At the early stages of oocyst wall formation, F-actin localization changed in alignment with the egg-shaped morphology of the forming oocysts with F-actin microfilaments making direct contact with the WFB1s. In tissue oocysts, the labelled actin cytoskeleton was situated underneath the forming outer layer of the oocyst wall. Treatment of macrogametocytes *in vitro* with the actin depolymerizing agents, Cytochalasin D and Latrunculin, led to a reduction in the numbers of mature WFB1s in the cytoplasm of the developing macrogametocytes, indicating that the actin plays an important role in WFB1 transport and oocyst wall formation in *E. maxima*.

Key words: actin, cytoskeletal inhibitors, WFB1 vesicle transport, *Eimeria* oocyst, 3D confocal microscopy.

INTRODUCTION

Parasites of the genus *Eimeria* cause chicken coccidiosis resulting in significant economic losses worldwide. *Eimeria* has been used as a model to study macrogametocyte maturation and oocyst wall biogenesis due to the fact that the sexual stages are easily harvested from the infected chicken intestine. Accordingly, numerous microscopic and molecular approaches have been utilized over the last several decades to characterize the basic aspects of the developmental biology of maturing male (micro-) and female (macro-) gametocytes (Mehlhorn, 1971; Pittilo and Ball, 1979; Ferguson *et al.* 2003). This research includes studies on the morphology and biochemical make-up of the two types of wall-forming bodies (Fried *et al.* 1992; Belli *et al.* 2002, 2004; Mouafo *et al.* 2002; Frölich *et al.* 2013) as well as the fine structure and biochemistry of the oocyst wall (Belli *et al.* 2009; Mai *et al.* 2009, 2011; Bushkin *et al.* 2013, 2012; Katrib *et al.* 2012; Rieux *et al.* 2012; Matsubayashi *et al.* 2014).

The process of oocyst wall formation involves the transport of wall-forming bodies (WFBs) to the parasite’s cell surface where the type 1 wall-

forming bodies (WFB1s) exocytose their cargo neutral (acid-fast) lipids into the outer layer of the oocyst wall (Bushkin *et al.* 2013; Frölich *et al.* 2013), while the WFB2s give rise to the inner oocyst wall containing cross-linked, tyrosine-rich glycoproteins (Belli *et al.* 2002, 2003), respectively. These molecules that are delivered to the developing oocyst wall are processed using an array of enzymes, including polyketide synthase (Bushkin *et al.* 2013), peroxidase and subtilisin (Belli *et al.* 2006; Katrib *et al.* 2012), glucan synthase and hydrolase (Bushkin *et al.* 2012). As a result, a waxy, lipid-rich outer oocyst wall is produced along with an inner wall layer composed of di-tyrosine cross-links and a trabecular scaffold of β -1, 3-glucans.

Scholtyssek and Mehlhorn proposed in the 1970s the concept that the transport of cargo lipids, sugars and proteins contained within the type 1 and 2 WFBs is mediated by the parasite’s ‘tubules’, each having a width of 75 Å (Mehlhorn, 1971). However, since that report, little work has been done to elucidate the involvement of the parasite’s cytoskeleton in regulating the changes in macrogametocyte morphology during oocyst wall formation and biogenesis.

In the present study, three-dimensional (3D) scanning laser confocal microscopy was employed using probes specific to the macrogametocyte WFBs type 1 and the actin cytoskeleton for studying macrogametocyte and oocyst differentiation at various developmental time points. Results from this study

* Corresponding author. The iThree Institute, School of Medical and Molecular Biosciences, University of Technology Sydney, PO Box 123, Broadway, Sydney, New South Wales 2007, Australia. Tel: +61-2-9514-8283. Fax: +61-2-9514-4026. E-mail: Sonja.Frolich@uts.edu.au

indicated that the actin cytoskeleton is directly involved in WFB1 transport to the periphery of the parasite during oocyst biogenesis. Furthermore, studies using inhibitors of actin polymerization to treat macrogametocytes *in vitro* indicated that both WFB1 integrity and oocyst wall biogenesis is dependent upon an intact actin cytoskeleton.

MATERIALS AND METHODS

Parasite maintenance and gametocyte harvest

The Houghton strain of *Eimeria maxima* used throughout these experiments was originally provided by Professor Martin Shirley (Institute for Animal Health, Compton, Newbury, Berkshire, UK). The oocysts were periodically passaged in 4-week-old Australorp chickens, and cleaned as described previously (Wagenbach *et al.* 1966). Gametocytes were prepared using techniques published previously (Frölich *et al.* 2014) with a slight modification. Briefly, chickens were infected at 4 weeks of age with 10 000 sporulated oocysts *per os*. They were sacrificed 132–134 h post-infection (pi) and their intestines were removed and immediately washed with ice-cold phosphate-buffered saline (PBS; pH 7.4). The intestines were slit open lengthwise and the mucosa scraped using a glass coverslip and the contents collected. The collected material, containing all stages of gametocytes development, including oocyst wall formation, was placed on the top of a 17 μm polymon filter and washed with PBS at room temperature. The material on the filter was discarded and the flowthrough, containing the gametocytes, was filtered through a 10 μm polymon filter. The gametocytes which accumulated on the filter were washed with PBS and counted in a counting chamber. The average yield obtained was in the range of $0.5\text{--}1 \times 10^5$ gametocytes/infected intestine.

Actin staining with phalloidin-Alexa514

Isolated sexual stages were fixed with 3% formaldehyde in SAC buffer (170 mM NaCl, 10 mM Tris pH 7, 10 mM glucose, 5 mM CaCl₂, pH 7.4) at room temperature for 15 min. After three washing steps in SAC buffer, the cells were resuspended in 1 ml of SAC containing 1% Triton X-100 and incubated at room temperature for 5 min to allow Permeabilization. Permeabilized cells were washed with SAC buffer, resuspended in 100 μL phalloidin-Alexa514 (1/100 dilution, Life technologies 30/32 Compark Circuit, Melbourne VIC 3170) and incubated for 45–60 min at room temperature. The cells were washed twice by centrifugation (1000 g, 5 min) and macrogametocyte wall forming bodies type 1 (WFB1s) and the outer oocyst wall material were visualized by incubating parasites in the presence of Evans blue, as described previously

(Ferguson *et al.* 2003; Frölich *et al.* 2014). Finally, samples were mounted on a glass slide with Vecta-Shield anti-fade medium (Vector Laboratories) and analysed by confocal microscopy.

In vitro experiments with drugs

Isolated gametocytes were immediately incubated with microfilament disorganizing drugs Cytochalasin D (Cyt D; Sigma, cat no: C8273) or Latrunculin (LAN; Sigma, cat no: L5163) dissolved in 0.1% dimethyl sulfoxide (DMSO) in SAC buffer (170 mM NaCl, 10 mM Tris pH 7, 10 mM glucose, 5 mM CaCl₂, pH 7.4). Each inhibitor was tested at 1 μM concentrations selected according to previous studies in protozoan parasites (Castillo-Romero *et al.* 2009, 2010; Angrisano *et al.* 2012; Herrera-Martinez *et al.* 2013; Luo *et al.* 2013). 0.1% DMSO, the drug diluents, was used as negative control. The experiments were performed in triplicate. All treatments were carried out for 30 min for 37 °C.

Confocal microscopy, image analysis and processing

A Nikon A1 laser scanning confocal microscope (Nikon) equipped with a 100 \times oil immersion lens (NA 1.4) objective, blue (ex 405, em 425–475 nm), green (ex 488, em 500–50 nm) and red filter sets (em 640 nm, ex 662–737 nm), NIS Elements software was used to collect image stacks with 0.2–0.5 μm distance between single planes. The *z*-projections were generated using Bitplane Imaris suite version 7.1.0 (Bitplane Scientific). The ‘spheres’ module was used to determine the number of the Evans blue-stained WFB1s in untreated and drug-treated parasites.

Statistical analysis

Quantitation of WFB1s was performed by randomly selecting ten microscopic fields of view at 100 \times magnification. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data are expressed as means \pm se unless otherwise stated. Data from two groups were compared using Student’s 2-tailed *t*-test for paired samples and data between groups were compared using a one-way analysis of variance (ANOVA). Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Three stages of oocyst wall assembly

Oocyst wall assembly during maturation of *Eimeria* macrogametocytes occurs by incorporation of precursors transported by the WFBs to the plasma membrane of the parasite. To follow the incorporation of WFB precursors, we used Evans blue stain as a

marker of WFB1 vesicles and the outer oocyst wall (Ferguson *et al.* 2003). We performed 3D confocal microscopy to analyse the spatial distribution of different steps of this process. This showed that three distinct stages in oocyst biogenesis could readily be distinguished in the isolated gametocytes: synthesis of the WFB1 vesicles, exocytosis of the precursors stored within the WFB1 vesicles and incorporation of the released material to form the outer oocyst wall (Fig. 1, supplementary Movies S1, S2).

Initially, oocyst wall precursors can be detected in the cytoplasm of gametocytes at around 132 h p.i. when the first cells in the population start to synthesize large WFB1 vesicles. As can be seen in Fig. 1, the top view (x -, y -, z -axis) of a rounded mature macrogametocyte (Fig. 1A) shows a total of 37 Evans-blue positive WFB1s located in the cytoplasm of the parasite. At this stage, WFB1 appear as large vesicles reaching $2.5\ \mu\text{m}$ in diameter (Fig. 1, supplementary Fig. S1A2). The number of WFB1 vesicles varies from cell to cell and is in the range of approximately 15–40. The top view in Fig. 1, supplementary Fig. S1A1, and the side view in Fig. 1, supplementary Fig. S2A2, of the WFB1s revealed narrow bridges extending laterally from each organelle linking adjacent WFB1s together (Fig. 1, supplementary Fig. S1A2 and arrows in S1A3). The surfaces of these organelles were nodular in appearance (Fig. 1, supplementary Fig. S1A3).

The top view of an early oocyst (Fig. 1B) revealed an ovoid parasite with approximately 23 WFB1s grouped in patches, presumably undergoing exocytosis. At this point, most of the WFB1s reached a maximum of $5\ \mu\text{m}$ in diameter, were located in the periphery, were fused together and were flattened on their surface revealing a homogeneous content (Fig. 1, supplementary Fig. S1B1–B2, arrows in B3). Statistical analysis of Evans blue positive WFB1 showed that, in mature macrogametocytes, the number of WFB1s with a minimum $2.5\ \mu\text{m}$ in diameter was 29.20 ± 2.878 . The Evans blue labelled outer oocyst wall of formed tissue oocysts showed that the wall material was patchy in appearance with areas that were unstained by the dye (Fig. 1C, arrows). Based on isosurface rendering (Fig. 1, supplementary Fig. S1C1) we observed nodules on the surface and areas of patchiness where the wall was still being formed (Fig. 1, supplementary Fig. S1C2 and arrows in S1C3). The 3D shape and the spatial distribution of WFB1s at various developmental stages are also shown in supplementary Movies S1–S2, which enables visualization of the parasites from all angles.

Morphological changes in the oocyst biogenesis involves actin remodelling

We used the fluorescent probe phalloidin to analyse the localization of polymerized F-actin in the cytoplasm of macrogametocytes at various

developmental time points. Evans blue-stained macrogametocytes were subsequently incubated with fluorescent phalloidin and analysed by two-dimensional (2D) and 3D confocal microscopy. As shown in Fig. 2A, phalloidin localized to microfilaments of various length comprising the fibrous network in the cytoplasm of mature macrogametocytes (Fig. 2, supplementary Fig. S2A, arrows). The microfilaments ranged from 3 to $5\ \mu\text{m}$ in length. At this stage, F-actin was seen linking the WFB1s into structures that resemble ‘beads-on-a-string’ (Fig. 2, supplementary Fig. S2A, arrows). Figure 2B and C shows in 3D the labelled F-actin forming a complex web-like meshwork in the cytoplasm of mature macrogametocytes with WFB1s trapped inside. Figure 2D shows a zoomed in top view of a 3D reconstruction, a corresponding single focal slice (Fig. 2, ‘2D’) and the corresponding isosurface rendering (Fig. 2, ‘isosurface’). It can be seen that labelled F-actin filaments are in direct contact with the WFB1s (Fig. 2, supplementary Fig. S2D, arrows). Double arrows in Fig. 2D illustrate fusion taking place between the WFB1s vesicles.

In mature macrogametocytes which are commencing oocyst wall formation (Fig. 3), all of the Evans Blue-stained WFB1s were situated in the periphery of the parasite (Fig. 3, A). The phalloidin-stained microfilaments were localized at the cell surface in alignment with the shape of the egg-like, early stage oocyst (Fig. 3, supplementary Fig. S3A, arrows). At that stage, the central cytoplasm was mostly free from microfilaments. Analysis of 3D micrographs showed the threads connecting the WFB1s to the microfilaments located at the cell periphery (Fig. 3, supplementary Fig. S3B–C). It can be seen that the labelled F-actin filaments are making direct contact with the WFB1s (Fig. 3, supplementary Fig. S3D, arrows), while several of the WFB1s had undergone fusion and released their contents into the outer layer of the forming oocyst wall (Fig. 2, double arrows in ‘2D’). The distribution of the actin cytoskeleton and the WFB1s at this stage of development is shown in the 3D reconstructions in supplementary material Movie S3 and S4.

Spatial reorganization of the actin cytoskeleton during oocyst biogenesis

In tissue oocysts, we observed the completed exocytosis of the WFB1s to form the outer oocyst wall (Fig. 4, supplementary Fig. S4A). At that point in parasite development, F-actin labelled microfilaments were not found in the cytoplasm of maturing oocysts (Fig. 4, supplementary Fig. S4A). Instead, the F-actin was found lying directly underneath and running in parallel with the formed outer layer of the oocyst wall (Fig. 4, arrows in supplementary Fig. S4A).

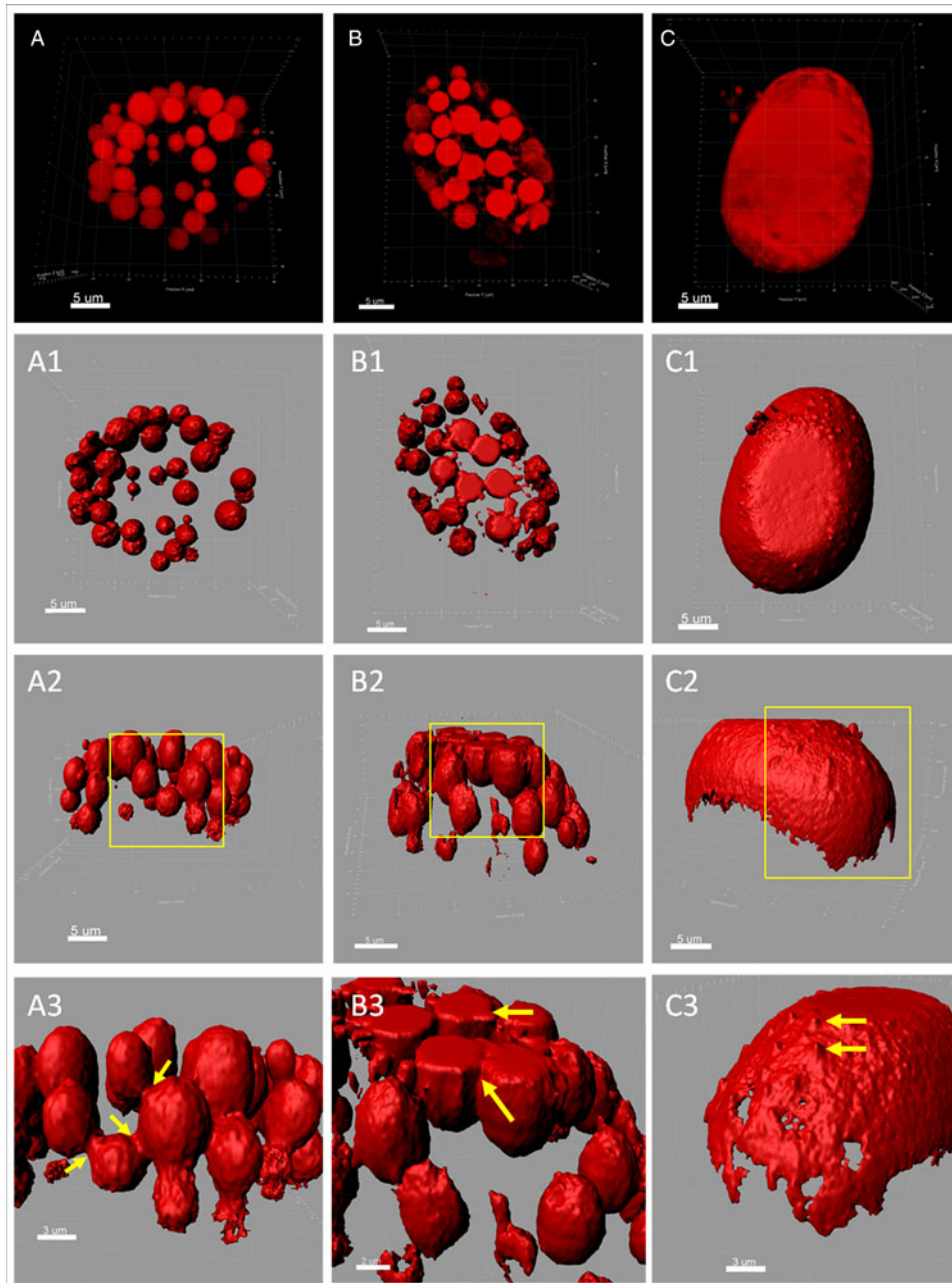


Fig. 1. The spatial distribution of WFB1s and the outer oocyst wall in macrogametocytes at different time points of oocyst wall biogenesis. (A, B, C) 3D projections with deconvolution of *E. maxima* mature macrogametocytes (A), early stage (B) and unsporulated oocysts (C) showing WFB1s and the outer oocyst wall (labelled in red). Quadrants correspond to 10 μ m. (A1, B1, C1) Top and side (A2, B2, C2) views of same samples as shown in A, B, C generated using the Isosurface tool in Imaris. Labelling shows 37 WFB1s with more than 80% of them situated in the periphery of mature macrogametocyte. In late-stage macrogametocytes, a total of 23 very large WFB1s in macrogametocytes at early stages of oocyst wall formation, and a full staining of the outer oocyst wall in unsporulated oocysts. (A3, B3, C3) Magnification of the regions depicted in A2, B2, C2. Scale bars are indicated.

In top views (x, y, z -axis) of 3D computer reconstructions, intense F-actin fluorescence was observed at the cell surface (Fig. 4, supplementary Fig. S4B and C). The web-like meshwork could no longer be observed in the cytoplasm at this stage of oocyst development. Instead, F-actin was mostly found at the surface of the parasite co-localizing with the outer oocyst wall (Fig. 4, supplementary Fig. S4B).

Figure 4C and D shows that the actin cytoskeleton was located underneath the outer oocyst wall in the periphery of the egg shaped parasite. At places where the wall was incomplete, we were able to observe the actin running underneath the forming oocyst wall (Fig. 4, supplementary Fig. S4D, arrows). The distribution of the actin cytoskeleton and the WFB1s can be also visualized in the 3D reconstructions shown in supplementary material, Movie S5.

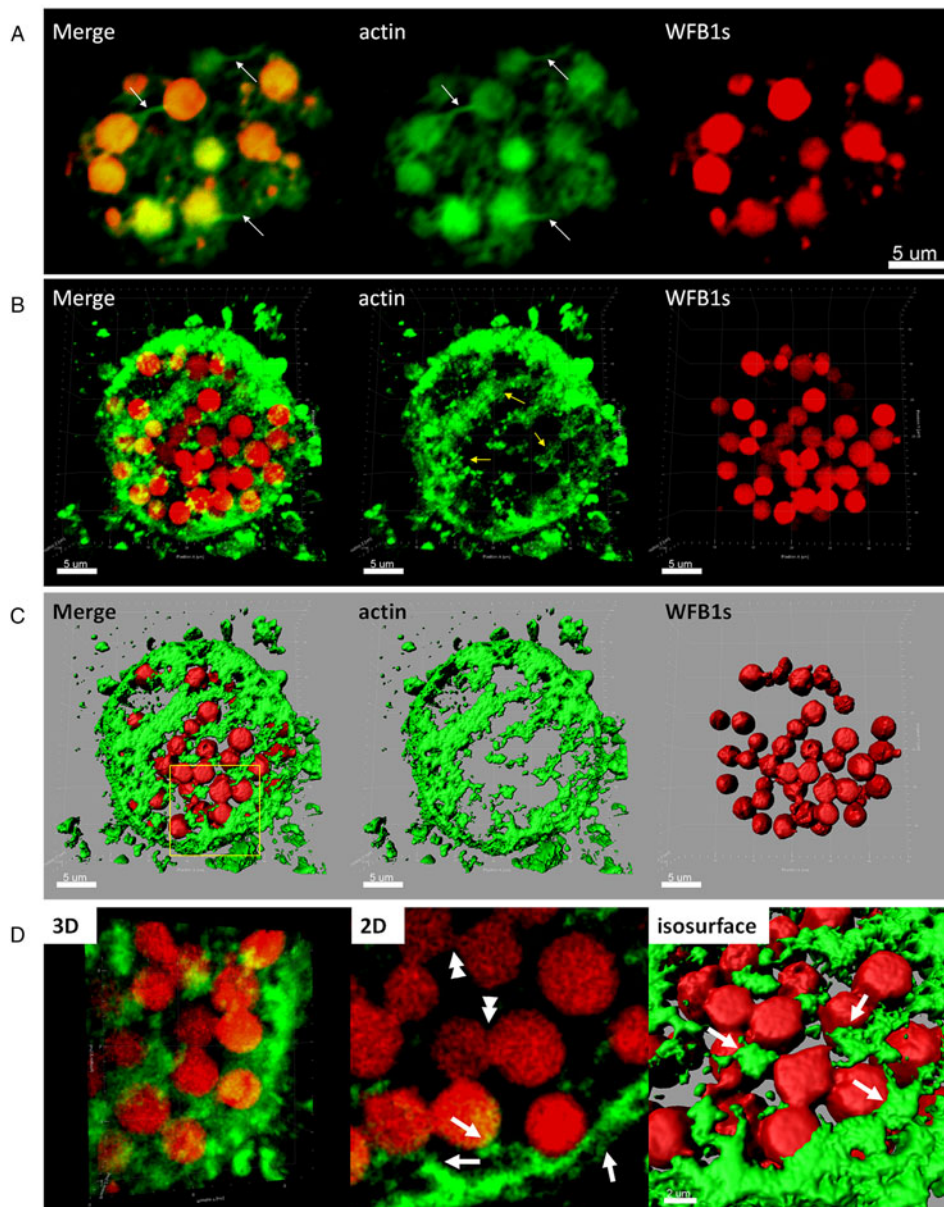


Fig. 2. Spatial distribution of actin and WFB1s in mature *E. maxima* macrogametocytes. (A) Conventional 2D confocal scanning laser microscopy of mature *E. maxima* macrogametocytes labelled with markers for F-actin (green) and for WFB1s (red). Note the labelling of long F-actin filaments physically connected to the WFB1s, (B) 3D reconstruction, (C) isosurface rendering, (D) magnification of the region depicted in C with corresponding 2D focal slice and surface rendering. Single arrows mark areas where the F-actin threads are physically associated with the WFB1s linking them together. Arrowheads point to WFB1s undergoing fusion. Scale bar is indicated.

Effects of actin inhibitors

Having observed the rearrangement of the actin cytoskeleton over the course of oocyst biogenesis in *E. maxima*, we investigated whether the F-actin participates in the transport of WFB1s during this process. Freshly extracted parasites were first tested for viability using Trypan blue exclusion (data not shown). They were then incubated in the presence of Cyt D, an inhibitor of actin filament polymerization and elongation (Selden *et al.* 1980; Goddette and Frieden, 1986), and LAN, which causes depolymerization of actin filaments (Morton *et al.* 2000). Following a 30 min incubation period

with these drugs, confocal microscopy was used to analyse the F-actin distribution, the number of formed WFB1s and the morphological appearance of formed tissue oocysts. Macrogametocytes incubated in 0.1% DMSO in SAC only were used as a negative control. As can be seen in Fig. 5, control macrogametocytes showed numerous large WFB1s and cytoplasmic actin filaments (Fig. 5, supplementary Fig. S5A, arrows). Macrogametocytes treated with Cyt D and LAN presented irregular morphology and structures of F-actin outlining the cytoplasmic membrane without forming the characteristic cytoplasmic filaments of untreated

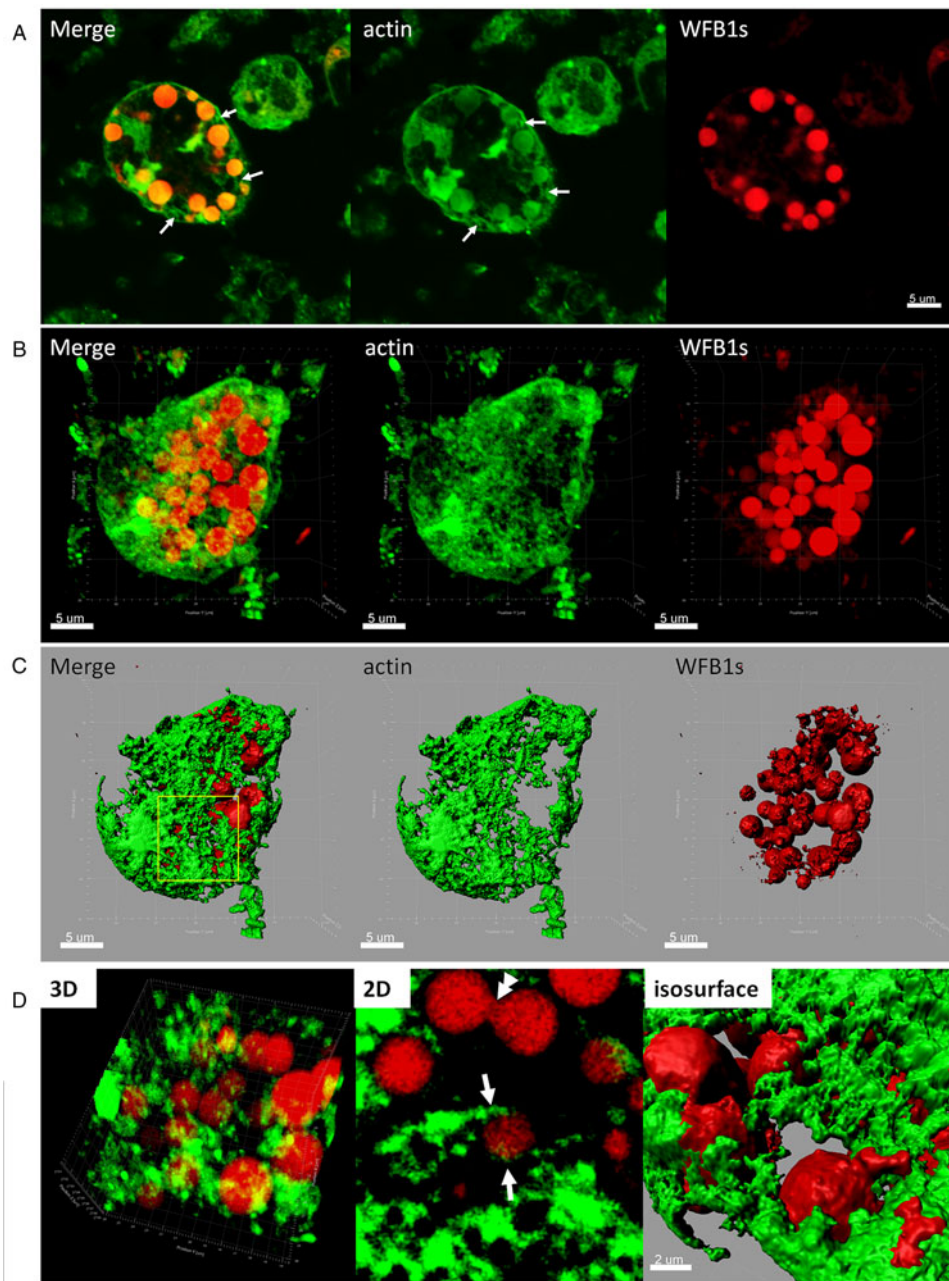


Fig. 3. Structure and organization of actin filaments and WFB1s in late stage *E. maxima* macrogametocytes. Confocal micrographs of late-stage macrogametocyte at early stages of oocyst wall formation labelled with Oregon Green phalloidin (green) and Evans blue (red). (A) Single cross-sections from 3D reconstruction of late macrogametocyte. Arrows mark the extensions of F-actin concentrated in the periphery of the parasite associated with large WFB1s vesicles linking them together to resemble ‘beads on a string’. (B, C) 3D reconstructions and projections of (A). (D) Magnification of the region depicted in (C) with corresponding 2D focal slice and isosurface rendering. Scale bars indicated.

macrogametocytes (Fig. 5, supplementary Fig. S5B and C, arrows).

In order to quantitate the effect of the drugs on maturing macrogametocytes, the total number of WFB1s was counted in ten drug-treated *vs* ten control parasites, which were randomly selected. As can be seen in Fig. 6A, untreated cells had an average of 29 ± 2.87 WFB1s [with a diameter of at least $2.5 \mu\text{m}$; a characteristic of fully formed WFB1s (Mehlhorn, 1971; Ferguson *et al.* 2003; Frölich *et al.* 2013)) per macrogametocyte,

whereas in the presence of Cyt D and LAN, the number of mature WFB1s was found to be significantly lower (13 ± 1.26 for Cyt D and 11 ± 1.2 for LAN WFB1s on average per parasite, $P < 0.001$). Corresponding examples of drug-treated and control macrogametocytes is shown in Figure 6B. Furthermore, oocysts in the drug-treated parasite suspension appeared misshapen and displayed collapsed oocyst walls, suggesting an alteration in oocyst wall formation (Fig. 6, supplementary Fig. S6B).

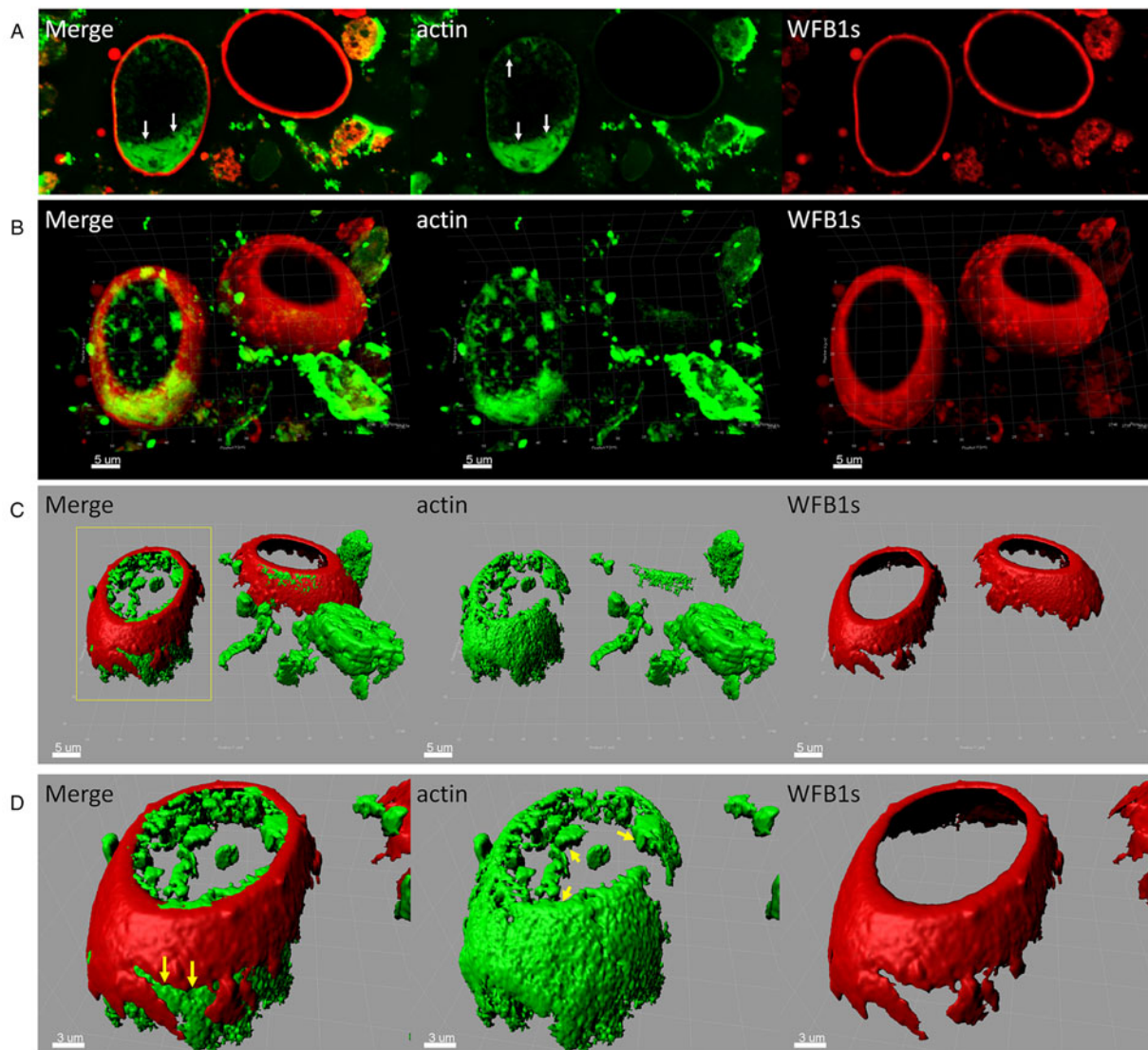


Fig. 4. The spatial organization of actin cytoskeleton and outer oocyst wall in *E. maxima* tissue cysts. (A) 2D and (B) 3D confocal microscopy of forming oocysts double stained with phalloidin (green) and Evans blue (red) showing the surface of the forming outer oocyst wall. Lying directly underneath and running in parallel is actin cytoskeleton (green). (C) Computer reconstructions of samples shown in (B). Rotations of the 3D reconstructions are presented in supplementary material Movie 5 and 6. (D) A zoomed view of the oocyst in (C), highlighting the patchiness of the outer oocyst wall in the process of formation. Scale bars indicated.

DISCUSSION

In this paper, we describe the role of the actin cytoskeleton in *E. maxima* macrogametocyte development and oocyst wall formation. Using markers specific for WFB1s and F-actin, which were visualized using confocal microscopy, we were able to demonstrate changes in the localization and distribution of F-actin at various stages of macrogametocyte maturation and oocyst biogenesis.

In previous studies, electron microscopy was used to observe mature macrogametocytes, and structures resembling 'tubules' were identified. It was hypothesized that these tubules may be involved in the transport of vesicles in maturing gametocytes (Mehlhorn, 1971). In the current study using fluorescent dye markers for F-actin and WFB1s, the actin

cytoskeleton organizational changes along with WFB1 localization were observed in freshly harvested gametocytes and early stage oocysts by 3D confocal microscopy. Our results showed that F-actin is in direct contact with WFB1s during their movement from the centre of the parasite to the periphery over the course of oocyst biogenesis. These results indicated that the transport of WFB1s is being actively facilitated by the actin filaments.

We next studied the subcellular localization and size of the actin microfilaments at various time points in macrogametocyte and oocyst development. We observed that in mature macrogametocytes the actin filaments were short and linked the WFB1s together into 'beads on a string'. This bead-like arrangement of the WFB1 vesicles appears to be

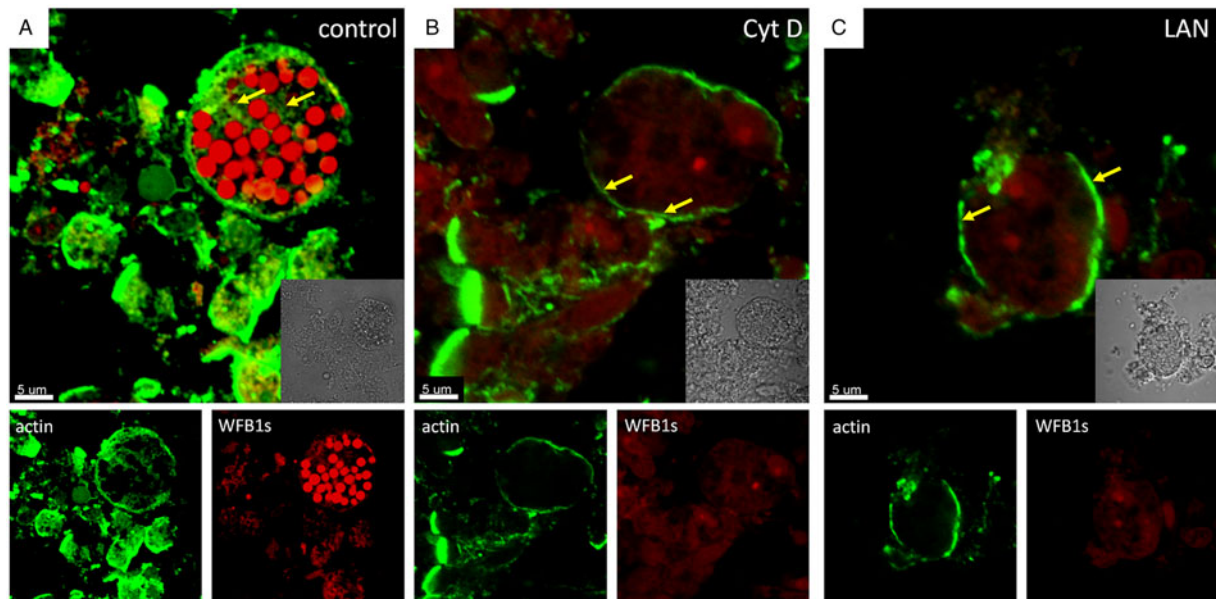


Fig. 5. Overall actin and WFB1s organization in *E. maxima* sexual stages treated with actin inhibitors. Freshly harvested *E. maxima* sexual stages were incubated with drugs, washed and fixed and F-actin and WFB1s were stained with Oregon Green-phalloidin (green) and Evans blue (red), respectively. 2D confocal micrographs show representative images of control (untreated; A) *E. maxima* macrogametocytes and those treated with Cyt D (B) and LAN (C), respectively. All treatments were carried out at 37 °C for 30 min. Scale bar represents indicated. Note the lack of actin filaments in the cytoplasm of drug-treated parasites and faint labelling of cytosolic WFB1s.

typical in mature macrogametocytes (Frölich *et al.* 2013). In addition, we found that the actin microfilaments formed a complex web-like meshwork throughout the cytoplasm of the mature macrogametocytes. As development continued to oocyst wall formation, the actin microfilaments were found to be mainly localized at the periphery of the parasite underneath the outer layer of the oocyst wall and that the microfilaments were found to be shorter in length. There is little if any actin remaining in the cytoplasm of the parasite at this stage of development. Throughout this process we were able to discern that the actin filaments were in direct contact with the WFB1s. From the observations in this study, we postulate that the actin microfilaments act to transport the WFB1s to the periphery of the parasite and once they reach that location they form direct bridges between themselves as they unload their cargo into the forming outer oocyst wall. These results indicate that actin may act to help form and stabilize the oocyst wall once the process of wall formation has been completed.

The importance of actin in oocyst biogenesis was shown by inhibitor studies with Cyt D and LAN. Both of these drugs have been used extensively to block the transport function of the actin cytoskeleton (Simon and Pon, 1996) as well as prevent cytoplasmic streaming. Both Cyt D and LAN treatment resulted in a significant reduction in the size and number of the WFB1s and abnormalities in the shape and size of the oocyst wall. These results

indicate that actin is essential for both the transport and maturation of the WFB1s, and is required for the synthesis of a properly formed oocyst.

In two other model cyst-forming parasites, *Giardia* and *Entamoeba*, actin was also shown to play an important role in encystation based on the morphological changes and decreased cyst yield caused by the drugs Cyt D, LAN and Jasplakinolide (Makioka *et al.* 2001; Castillo-Romero *et al.* 2009, 2010). Like in *Eimeria*, these parasites have encystation specific secretory vesicles containing β glucans, Cyst wall proteins (COWP), GalNac, acid-fast/neutral lipids and enzymes such as glucan synthases and cysteine proteases (Ellis *et al.* 1996; Hetsko *et al.* 1998; Bulik *et al.* 2000; Gerwig *et al.* 2002; Lanfredi-Rangel *et al.* 2003; Chavez-Munguia *et al.* 2004; De Souza, 2006; Ebert *et al.* 2008; Chatterjee *et al.* 2010; Faso *et al.* 2013; Samuelson *et al.* 2013).

In *Eimeria*, oocyst biogenesis can be divided into three phases – early, mid and late. During the early phase, changes in structure, metabolism, gene expression and protein transport occur (Mouafo *et al.* 2002; Ferguson *et al.* 2003; Belli *et al.* 2009; Mai *et al.* 2009; Walker *et al.* 2010; Katrib *et al.* 2012; Frölich *et al.* 2013). The formation of WFB1s occurs in the mid phase (mature macrogametocytes). All of our results suggest that F-actin contributes to WFB1 formation, and that it participates in the mid to late stages by regulating WFB1 localization and transport to the periphery of the oocyst through the actin cytoskeleton. It will be

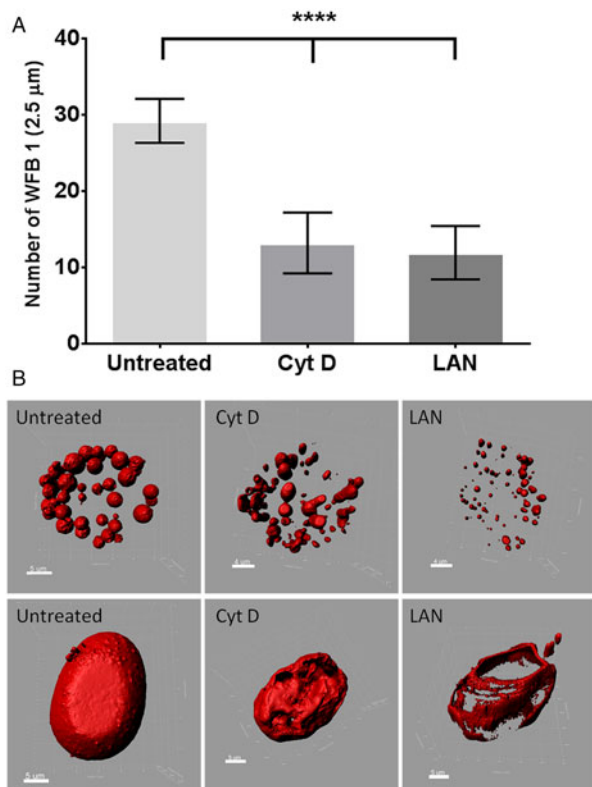


Fig. 6. Effect of actin cytoskeleton inhibitors on the maturation of WFB1s vesicles and the outer oocyst wall. The cells were treated with 1 μM Cyt D or 1 μM LAN for 30 min and stained with a marker for WFB1s (red). DMSO was used as a control (untreated). (A) Quantitative analysis of WFB1s with $\geq 2.5 \mu\text{m}$ in diameter. Results were analysed by one-way ANOVA comparison tests between the groups (untreated, Cyt D and LAN treated). Error bars are \pm S.E.M. $***P < 0.001$. (B) 3D confocal microscopy showing the shape and size of WFB1s and outer oocyst wall in untreated and treated parasites. Note the misshaped oocyst walls in drug-treated parasite suspension.

interesting to analyse how molecular motors, which have been identified in the *Eimeria maxima* genome (Blake *et al.* 2012; Reid *et al.* 2014) and gametocyte proteome (unpublished data) interact with the WFB1s and consider other possible proteins that may regulate WFB1 transport.

Based on the results of the present study, we concluded that the actin cytoskeleton plays a key role in the WFB1s transport and in differentiation of macrogametocytes into oocysts. Therefore, the molecules involved in the transport and proper processing of these organelles represent potential targets for the development of new transmission blocking strategies. Work is currently in progress to identify the relevant proteins involved in these key developmental processes.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit <http://dx.doi.org/S0031182015000207>.

ACKNOWLEDGEMENTS

We thank the team at the Ernst Facility for maintaining the chickens. Authors gratefully acknowledge the Faculty of Science, University of Technology, Sydney for funding this work. SF was supported by Australian Postgraduate Award. We are indebted to Associate Professor Cynthia Whitchurch (Director of the Microbial Imaging Facility at the iThree Institute, University of Technology Sydney) for inspiring discussions and her critical assessment of the work presented here. We thank Ms Erin Gloag (University of Technology, Sydney) for her help with statistical analysis.

REFERENCES

- Angrisano, F., Riglar, D. T., Sturm, A., Volz, J. C., Delves, M. J., Zuccala, E. S., Turnbull, L., Dekiwadia, C., Olshina, M. A., Marapana, D. S., Wong, W., Mollard, V., Bradin, C. H., Tonkin, C. J., Gunning, P. W., Ralph, S. A., Whitchurch, C. B., Sinden, R. E., Cowman, A. F., McFadden, G. I. and Baum, J. (2012). Spatial localisation of actin filaments across developmental stages of the malaria parasite. *PLoS ONE* 7, e32188.
- Belli, S. I., Lee, M., Thebo, P., Wallach, M. G., Schwartzburd, B. and Smith, N. C. (2002). Biochemical characterisation of the 56 and 82 kDa immunodominant gametocyte antigens from *Eimeria maxima*. *International Journal for Parasitology* 32, 805–816.
- Belli, S. I., Wallach, M. G., Luxford, C., Davies, M. J. and Smith, N. C. (2003). Roles of tyrosine-rich precursor glycoproteins and dityrosine- and 3,4-dihydroxyphenylalanine-mediated protein cross-linking in development of the oocyst wall in the coccidian parasite *Eimeria maxima*. *Eukaryotic Cell* 2, 456–464.
- Belli, S. I., Mai, K., Skene, C. D., Gleeson, M. T., Witcombe, D. M., Katrib, M., Finger, A., Wallach, M. G. and Smith, N. C. (2004). Characterisation of the antigenic and immunogenic properties of bacterially expressed, sexual stage antigens of the coccidian parasite, *Eimeria maxima*. *Vaccine* 22, 4316–4325.
- Belli, S. I., Smith, N. C. and Ferguson, D. J. P. (2006). The coccidian oocyst: a tough nut to crack! *Trends in Parasitology* 22, 416–423.
- Belli, S. I., Ferguson, D. J. P., Katrib, M., Slapetova, I., Mai, K., Slapeta, J., Flowers, S. A., Miska, K. B., Tomley, F. M., Shirley, M. W., Wallach, M. G. and Smith, N. C. (2009). Conservation of proteins involved in oocyst wall formation in *Eimeria maxima*, *Eimeria tenella* and *Eimeria acervulina*. *International Journal for Parasitology* 39, 1063–1070.
- Blake, D. P., Alias, H., Billington, K. J., Clark, E. L., Mat-Isa, M. N., Mohamad, A. F., Mohd-Amin, M. R., Tay, Y. L., Smith, A. L., Tomley, F. M. and Wan, K. L. (2012). EmaxDB: availability of a first draft genome sequence for the apicomplexan *Eimeria maxima*. *Molecular and Biochemical Parasitology* 184, 48–51.
- Bulik, D. A., van Ophem, P., Manning, J. M., Shen, Z. J., Newburg, D. S. and Jarroll, E. L. (2000). UDP-N-acetylglucosamine pyrophosphorylase, a key enzyme in encysting *Giardia*, is allosterically regulated. *Journal of Biological Chemistry* 275, 14722–14728.
- Bushkin, G. G., Motari, E., Magnelli, P., Gubbels, M.-J., Dubey, J. P., Miska, K. B., Bullitt, E., Costello, C. E., Robbins, P. W. and Samuelson, J. (2012). Beta-1,3-Glucan, which can be targeted by drugs, forms a trabecular scaffold in the oocyst walls of *Toxoplasma* and *Eimeria*. *Mbio* 3, e00258–e00312.
- Bushkin, G. G., Motari, E., Carpentieri, A., Dubey, J. P., Costello, C. E., Robbins, P. W. and Samuelson, J. (2013). Evidence for a structural role for acid-fast lipids in oocyst walls of *Cryptosporidium*, *Toxoplasma*, and *Eimeria*. *Mbio* 4, e00387–e00313.
- Castillo-Romero, A., Leon-Avila, G., Perez Rangel, A., Cortes Zarate, R., Garcia Tovar, C. and Hernandez, J. M. (2009). Participation of actin on *Giardia lamblia* growth and encystation. *PLoS ONE* 4, e7156.
- Castillo-Romero, A., Leon-Avila, G., Wang, C. C., Perez Rangel, A., Camacho Nuez, M., Garcia Tovar, C., Ayala-Sumano, J. T., Luna-Arias, J. P. and Hernandez, J. M. (2010). Rab11 and actin cytoskeleton participate in *Giardia lamblia* encystation, guiding the specific vesicles to the cyst wall. *PLoS Neglected Tropical Diseases* 4, e697.
- Chatterjee, A., Carpentieri, A., Ratner, D. M., Bullitt, E., Costello, C. E. *et al.* (2010). *Giardia* cyst wall protein 1 is a Lectin that binds to curled fibrils of the GalNAc homopolymer. *PLoS Pathog* 6(8): e1001059. doi:10.1371/journal.ppat.1001059.
- Chavez-Munguia, B., Cedillo-Rivera, R. and Martinez-Palomo, A. (2004). The ultrastructure of the cyst wall of *Giardia lamblia*. *Journal of Eukaryotic Microbiology* 51, 220–226.

- De Souza, W. (2006). Secretory organelles of pathogenic protozoa. *Anais Da Academia Brasileira De Ciencias* **78**, 271–291.
- Ebert, F., Bachmann, A., Nakada-Tsukui, K., Hennings, I., Drescher, B., Nozaki, T., Tannich, E. and Bruchhaus, I. (2008). An *Entamoeba* cysteine peptidase specifically expressed during encystation. *Parasitology International* **57**, 521–524.
- Ellis, J. E., Wyder, M. A., Jarroll, E. L. and Kaneshiro, E. S. (1996). Changes in lipid composition during *in vitro* encystation and fatty acid desaturase activity of *Giardia lamblia*. *Molecular and Biochemical Parasitology* **81**, 13–25.
- Faso, C., Bischof, S. and Hehl, A. B. (2013). The proteome landscape of *Giardia lamblia* encystation. *PLoS ONE* **8**(12): e83207. doi:10.1371/journal.pone.0083207.
- Ferguson, D. J. P., Belli, S. I., Smith, N. C. and Wallach, M. G. (2003). The development of the macrogamete and oocyst wall in *Eimeria maxima*: immuno-light and electron microscopy. *International Journal for Parasitology* **33**, 1329–1340.
- Fried, M., Mencher, D., Sarshalom, O. and Wallach, M. (1992). Developmental gene-expression of 230-kilodalton macrogamete-specific protein of the avian coccidial parasite, *Eimeria maxima*. *Molecular and Biochemical Parasitology* **51**, 251–262.
- Frölich, S., Johnson, M., Robinson, M., Entzeroth, R. and Wallach, M. (2013). The spatial organization and extraction of the wall-forming bodies of *Eimeria maxima*. *Parasitology* **140**, 876–887.
- Frölich, S., Shahparee, A., Wasinger, V. C. and Wallach, M. (2014). *In vivo* localization of antibodies raised against *Eimeria maxima* wall forming bodies during sexual intracellular development. *Parasitology* (2014), **141**, 1726–1735, doi:10.1017/S0031182014001012.
- Gerwig, G. J., van Kuik, J. A., Leeftang, B. R., Kamerling, J. P., Vliegthart, J. F. G., Karr, C. D. and Jarroll, E. L. (2002). The *Giardia intestinalis* filamentous cyst wall contains a novel beta(1–3)-N-acetyl-D-galactosamine polymer: a structural and conformational study. *Glycobiology* **12**, 499–505.
- Goddette, D. W. and Frieden, C. (1986). Actin polymerization. The mechanism of action of cytochalasin D. *Journal of Biological Chemistry* **261**, 15974–15980.
- Herrera-Martínez, M., Hernández-Ramírez, V. I., Lagunes-Guillen, A. E., Chavez-Munguia, B. and Talamas-Rohana, P. (2013). Actin, RhoA, and Rab11 participation during encystment in *Entamoeba invadens*. *BioMed Research International* **2013**, 919345–919345.
- Hetsko, M. L., McCaffery, J. M., Svard, S. G., Meng, T. C., Que, X. C. and Gillin, F. D. (1998). Cellular and transcriptional changes during excystation of *Giardia lamblia* *in vitro*. *Experimental Parasitology* **88**, 172–183.
- Katrib, M., Ikin, R. J., Brossier, F., Robinson, M., Slapetova, I., Sharman, P. A., Walker, R. A., Belli, S. I., Tomley, F. M. and Smith, N. C. (2012). Stage-specific expression of protease genes in the apicomplexan parasite, *Eimeria tenella*. *BMC Genomics* **13**, 685–712.
- Lanfredi-Rangel, A., Attias, M., Reiner, D. S., Gillin, F. D. and De Souza, W. (2003). Fine structure of the biogenesis of *Giardia lamblia* encystation secretory vesicles. *Journal of Structural Biology* **143**, 153–163.
- Luo, W., Yu, C.-H., Lieu, Z. Z., Allard, J., Mogilner, A., Sheetz, M. P. and Bershadsky, A. D. (2013). Analysis of the local organization and dynamics of cellular actin networks. *Journal of Cell Biology* **202**, 1057–1073.
- Mai, K., Sharman, P. A., Walker, R. A., Katrib, M., De Souza, D., McConville, M. J., Wallach, M. G., Belli, S. I., Ferguson, D. J. P. and Smith, N. C. (2009). Oocyst wall formation and composition in coccidian parasites. *Memorias Do Instituto Oswaldo Cruz* **104**, 280–288.
- Mai, K., Smith, N. C., Feng, Z.-P., Katrib, M., Slapeta, J., Slapetova, I., Wallach, M. G., Luxford, C., Davies, M. J., Zhang, X., Norton, R. S. and Belli, S. I. (2011). Peroxidase catalysed cross-linking of an intrinsically unstructured protein via dityrosine bonds in the oocyst wall of the apicomplexan parasite, *Eimeria maxima*. *International Journal for Parasitology* **41**, 1157–1164.
- Makioka, A., Kumagai, M., Ohtomo, H., Kobayashi, S. and Takeuchi, T. (2001). Effect of jasplakinolide on the growth, encystation, and actin cytoskeleton of *Entamoeba histolytica* and *Entamoeba invadens*. *Journal of Parasitology* **87**, 399–405.
- Matsubayashi, M., Hatta, T., Miyoshi, T., Anisuzzaman, A., Sasai, K., Yamaji, K., Shimura, K., Isobe, T., Kita, K. and Tsuji, N. (2014). Localization of eimeripain, an *Eimeria tenella* cathepsin B-like cysteine protease, during asexual and sexual intracellular development in chicken ceca. *Journal of Veterinary Medical Science* **76**, 531–537.
- Mehlhorn, E. S. A. H. (1971). Fine structure of macrogametes and oocysts of coccidia and related organisms. *Zeitschrift Fur Parasitenkunde* **37**, 1–43.
- Morton, W. M., Ayscough, K. R. and McLaughlin, P. J. (2000). Latrunculin alters the actin-monomer subunit interface to prevent polymerization. *Nature Cell Biology* **2**, 376–378.
- Mouafo, A. N., Weck-Heimann, A., Dubremetz, J. F. and Entzeroth, R. (2002). Monoclonal antibodies specific for the two types of wall-forming bodies of *Eimeria tenella* macrogametes (Coccidia, Apicomplexa). *Parasitology Research* **88**, 217–224.
- Pittilo, R. M. and Ball, S. J. (1979). Fine structure of the developing macrogamete and oocyst wall formation in *Eimeria maxima*. *Parasitology* **79**, R34–R34.
- Reid, A. J., Blake, D. P., Ansari, H. R., Billington, K., Browne, H. P., Bryant, J., Dunn, M., Hung, S. S., Kawahara, F., Miranda-Saavedra, D., Malas, T. B., Mourier, T., Naghra, H., Nair, M., Otto, T. D., Rawlings, N. D., Rivailler, P., Sanchez-Flores, A., Sanders, M., Subramaniam, C., Tay, Y.-L., Woo, Y., Wu, X., Barrell, B., Dear, P. H., Doerig, C., Gruber, A., Ivens, A. C., Parkinson, J., Rajandream, M.-A. et al. (2014). Genomic analysis of the causative agents of coccidiosis in domestic chickens. *Genome Research* **24**, 1676–1685.
- Rieux, A., Gras, S., Lecaille, F., Niepceron, A., Katrib, M., Smith, N. C., Lalmanach, G. and Brossier, F. (2012). Eimeripain, a cathepsin B-like cysteine protease, expressed throughout sporulation of the apicomplexan parasite *Eimeria tenella*. *PLoS ONE* **7**, e31914.
- Samuelson, J., Bushkin, G. G., Chatterjee, A. and Robbins, P. W. (2013). Strategies to discover the structural components of cyst and oocyst walls. *Eukaryotic Cell* **12**, 1578–1587.
- Selden, L. A., Gershman, L. C. and Estes, J. E. (1980). A proposed mechanism of action of cytochalasin D on muscle actin. *Biochemical and Biophysical Research Communications* **95**, 1854–1860.
- Simon, V. R. and Pon, L. A. (1996). Actin-based organelle movement. *Experientia* **52**, 1117–1122.
- Wagenbach, G. E., Challey, J. R. and Burns, W. C. (1966). A method for purifying coccidian oocysts employing Chlorox and sulfuric acid-dochromate solution. *Journal of Parasitology* **52**, 1222.
- Walker, R. A., Slapetova, I., Slapeta, J., Miller, C. M. and Smith, N. C. (2010). The Glycosylation pathway of *Eimeria tenella* is upregulated during gametocyte development and may play a role in oocyst wall formation. *Eukaryotic Cell* **9**, 127–135.