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PRODUCTION OF *T. GONDII* TRANSGENIC STRAINS TO CHARACTERIZE THE TUBULIN COFACTOR B INTERACTOME

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Resumo

Toxoplasma gondii é um parasita intracelular obrigatório que infeta a maioria dos animais, apresentando uma distribuição mundial. Este apresenta duas fases no seu ciclo de vida: uma fase assexual (que ocorre no interior do hospedeiro intermediário) e uma fase sexual (que ocorre no interior do hospedeiro definitivo). O parasita apresenta uma morfologia eucariótica clássica, sendo uma célula altamente polarizada. De entre as suas estruturas, o complexo apical é uma estrutura essencial para a patogenicidade e sobrevivência do parasita, uma vez que apresenta várias estruturas (como o conoide e os microtúbulos sub-peliculares) que apresentam uma elevada importância ao nível da invasão da célula do hospedeiro. O cofator B da tubulina (TBCB) é uma proteína envolvida nas vias de *folding* e degradação da tubulina, com mecanismos mal caracterizados em *T. gondii*, e em que a sua expressão pode estar relacionada com as taxas de invasão e saída da célula. Por este motivo, o grande objetivo é caracterizar o interactoma do TBCB e para isto iremos utilizar o sistema de BioID, que permite usar biotinilação por proximidade das proteínas, para determinar os mecanismos de ação do TBCB. Para isso, pretendemos com este trabalho produzir estirpes transgénicas de *T. gondii* que expressem BirA em fusão com TBCB (BirA_TBCB) e apenas BirA.

Abstract

Toxoplasma gondii is an obligate intracellular parasite infecting almost all warm-blooded animals, with a world-wide distribution. It presents two phases on its life cycle: an asexual phase (within the intermediate host) and a sexual phase (within the definitive host). The parasite exhibits a classic eukaryotic morphology, being a high polarized cell. Among its structures, apical complex is an essential structure for parasite pathogenicity and survival, as it presents several structures (as conoid and sub-pellicular microtubules) that play an important role on host cell invasion. Tubulin cofactor B (TBCB) is a protein involved in α-tubulin folding and degradation pathway, with a poorly characterized mechanism, that its expression might be related to invasion and egress levels. For this reason, we aim to characterize the TBCB interactome and with that objective we pretend to use BioID method, which allows to use proximity dependent biotin identification of proteins, to determine TBCB mechanisms of action. For that, we aim to produce *T. gondii* transgenic strains expressing BirA in fusion with tubulin cofactor B and only BirA.

Key words: *Toxoplasma gondii*, tubulin cofactor B (TBCB), BioID

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Abbreviations

ATP – Adenosine triphosphate

DMEM – Dulbecco's Modified Eagle's Medium

GDP – Guanosine diphosphate

GTP – Guanosine triphosphate

HFF – Human foreskin fibroblasts

HRP – Horseradish peroxidase

MAP – Microtubule associated proteins

PBS – Phosphate buffered saline

PCR – Polimerase chain reaction

TAE – Tris-acetate-EDTA

TBCA – Tubulin cofactor A

TBCB – Tubulin cofactor B

TBCC – Tubulin cofactor C

TBCD – Tubulin cofactor D

TBCE – Tubulin cofactor E

PTM – post-translational modification

1. Introduction

1.1. *Toxoplasma gondii* and toxoplasmosis

Toxoplasma gondii is an obligate intracellular parasite infecting almost all warm-blooded animals, with a world-wide distribution. It presents a high medical an veterinary importance, and is used as a model for cell biology and molecular studies (Dubey, 2008).

Toxoplasmosis is a parasitic disease caused by *T. gondii*, and its considered as one of the most common parasitic zoonoses world-wide.

1.2. History

T. gondii was initially found in 1908 while Charles Nicolle and Louis Manceaux were working with tissues of hamster-like rodent, *Ctenodactylus gundi*, used for leishmaniasis research at the Pasteur Institute in Tunis. (Dubey, 2008). At first, scientists believed the parasite to be a piroplasm, then *Leishmania*, but later they realized they found a new organism (Dubey, 2008). In the same year (1908) in Brazil, a scientist discovered a similar parasite in rabbits. This parasite was named based on the morphology of the infectious stage (*Toxo*=arc*; plasma*=life) and the host (Dubey, 2008; Ferguson, 2009; Innes, 2010).

In the years that followed, many *T. gondii*-like organisms were identified in several other hosts. Sabin an Olitsky were able to isolate for the first time the first viable *T. gondii*, in 1937, using techniques previously employed in the study of viruses (Sabin & Olitsky, 1937). In 1939, Sabin proved that biological and immunological characteristics of different *Toxoplasma* isolates from animals and humans were identical to *T. gondii* (Sabin, 1939).

There is only one species in the genus *Toxoplasma*, being *T. gondii*. Even though this parasite has a worldwide distribution and presents the widest host range of all parasites (Dubey, 2008). For the following years, the research on this parasite increased substantially and important discoveries were made about its biology. *T. gondii*, being a parasite that is easily cultured in laboratory and has a wide range of animal models available, became a very accessible model organism for scientists (Innes, 2010).

1.3. Epidemiology

T. gondii is probably the most common parasite world-wide, infecting almost all warm-blooded animals. Its prevalence is high, being around 25-50% (Robert-gangneux & Dardé, 2012).

Most human population that becomes infected with *T. gondii* remains asymptomatic as the immune system usually keeps the parasite from causing illness. Worldwide seroprevalence of the parasite varies between 1-100% depending on the environmental and socioeconomic conditions, including eating habits and health-related practices. Lowest seroprevalence (~1%) is found in countries in Far East, and highest (>90%) is found in some parts of European and South American countries (Israili, Flegr, Prandota, & Sovic, 2014).

1.4. Life cycle

Approximately 60 years after its discovery, the complete life cycle of *T. gondii* was finally elucidated as scientists have found the central role of the member of *Felidae* family as definitive hosts for the sexual phase of parasites' development (Frenkel, Dubey, & Miller, 1970; Dubey and Beattie, 1988; Ferguson, 2009). *T. gondii* is a tissue-cyst-forming coccidium able to alternate between definitive, where sexual reproduction occurs, and intermediate hosts, where asexual replication occurs (Figure 1) Due to this, it was possible to explain the widespread infection in most warm-blooded animals, including humans, as intermediate hosts (Robert-gangneux & Dardé, 2012).

In felids, after ingestion of cysts present in tissues of an intermediate host, the cyst wall is destroyed by gastric enzymes. In the small intestine, bradyzoites initiate another asexual phase of proliferation consisting of initial multiplication by endodyogeny followed by repeated endopolygeny in epithelial cells. After that, both female and male gametes are formed (gametogony). Upon fertilization, oocysts formed within enterocytes are released as the cell is disrupted and are excreted as unsporulated forms in cat faeces. A few days later, the process of sporogony starts in the external environment, leading to the development of infectious oocysts. Infected felids can shed more than 100 million oocysts in their faeces, which mean they can infect a wide range of intermediate hosts when ingested (Tenter et al., 2000; Robert-Gangneux & Dardé, 2012).

Within intermediate hosts, the parasite undergoes only sexual development. The sporozoites are released after oocyst ingestion, and they penetrate the intestinal epithelium of the intermediate host, where they differentiate into tachyzoites. Tachyzoites multiply rapidly by repeated endodyogeny in different types of cells. Recently generated tachyzoites initiate the second phase of development resulting in the formation of tissue cysts. Within the tissue cysts, bradyzoites multiply slowly by endodyogeny. Cysts have a higher affinity for neural and muscular tissues, as they are predominantly located along

the central nervous system (CNS). However, they can also be present in the eye as well as skeletal and cardiac muscles. However, to a lesser extent may also be found in visceral organs, such as lungs, liver, and kidneys. Tissue cysts are the terminal life cycle stage in intermediate hosts, and in some species, somehow they can persist for the whole life of the host. As an answer to this mechanism, some investigators believe that tissue cysts break down periodically, with bradyzoites differentiating into tachyzoites that reinvade host cells and then form new tissue cysts (Reviewed by Tenter et al., 2000; Robert-Gangneux & Dardé, 2012).

The ingestion of tissue cysts by an intermediate host through raw or undercooked meat leads to cysts rupture as they pass through the digestive tract, releasing bradyzoites. This will infect the intestinal epithelium of the new host and differentiate into tachyzoites for dissemination throughout the body (Robert-Gangneux & Dardé, 2012).

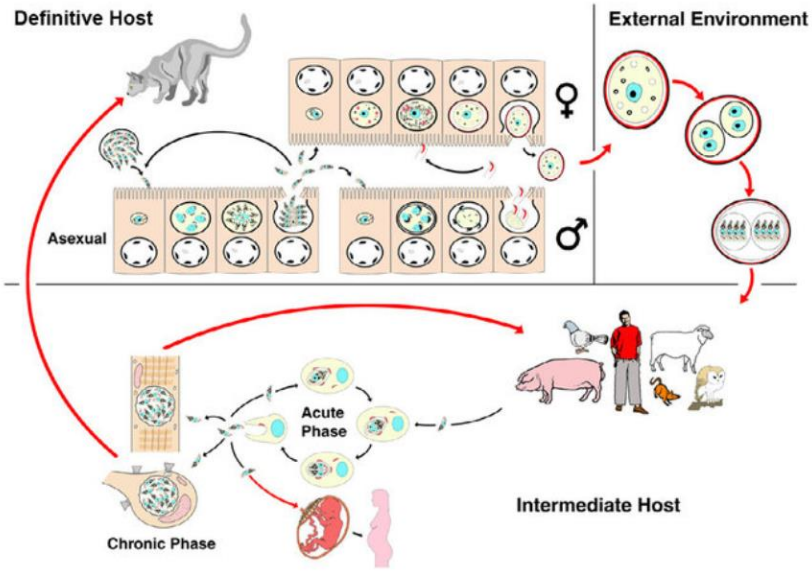

Figure 1 – Life cycle of *Toxoplasma gondii*. In felids, after oocyst ingestion, bradyzoites multiplicate forming gametes. Upon fertilization, oocysts are released into the environment, where they develop into infectious oocysts. When intermediate hosts ingest oocysts, sporozoites are released and differentiate into tachyzoites, which multiply rapidly causing the acute phase of infection, and differentiate into bradyzoites that form cysts (From (Freppel, Ferguson, Shapiro, & Dubey, 2019) with permission)

1.4.1. The lytic cycle of *T. gondii*

Has an obligate intracellular parasite, *T. gondii* lives within a nucleated host cell to grow and divide. However, tachyzoites spend time in the extracellular environment after egress from a host cell, seeking a new host cell to invade, perpetuating the lytic

cycle (Hortua Triana, Márquez-Nogueras, Vella, & Moreno, 2018). To maintain the propagation of the infection, the parasite needs invasion and egress, two highly regulated and dynamic processes. Through invasion, proteins from specific apical organelles are secreted, mediating attachment to the host cells and formation of the parasitophorous vacuole occupied by the parasite. (Ovciarikova, Lemgruber, Stilger, Sullivan, & Sheiner, 2017; Hortua Triana, Márquez-Nogueras, Vella, & Moreno, 2018).

1.4.1.1. Host cell invasion

Invading the host cell requires many steps, as it is a complex process. The invasion by *T. gondii* can be separated into two major steps: (1) cell recognition and binding, and (2) vacuole formation during entry (Li, Wang, Zhao, & Zhang, 2007; Sweeney, Morrissette, Lachapelle, & Blader, 2010).

T. gondii invasion is highly polarized, as the parasite uses exclusively its apical end to initiate penetration into the target cell. The release of proteins as MICs and ROPs, having an apical location, and GRAs, which are dispersed in the cell, from specialized secretory organelles allows the parasite to attach its apical region to the host cell, initiating the parasite gliding motility on the cell surface until it enters by penetrating the cell membrane (Zhang, Shiun, Juhas, & Zhang, 2019). The parasite penetration causes an invagination of the plasma membrane resulting in the formation of the parasitophorous vacuole, where the parasite will reside (Carruthers & Boothroyd, 2007; Sweeney et al., 2010).

1.4.1.2. Cellular Division

Apicomplexan parasites divide by different mechanisms from their hosts, as they replicate by the formation of daughter parasites within a fully polarized mother cell. There have been described three distinct mechanisms on the basis of extent and timing of nuclear division before cytokinesis: endodyogeny, schizogony and endopolygeny (Morrissette & Sibley, 2002; Gubbels, White, & Szatanek, 2008; Francia & Striepen, 2014).

Endodyogeny is an asexual reproduction process in which the parasite produces two daughter cells per mitotic cycle. On the other hand, endopolygeny is a similar process, but the parasite produces multiple daughter cells from a single mother cell.

These two mechanisms are used specially by *T. gondii* within the intermediate host (Hu et al., 2002; Morrissette & Sibley, 2002).

On the contrary, schizogony is an asexual reproduction process, that occurs in parasites like *Plasmodium sp.*, in which the parasite undergoes multiple rounds of nuclear division without cytoplasmatic division, followed by a segmentation of the mother cell to create progeny. However, some authors describe the asexual reproduction processes from *T. gondii* as being a variation of schizogony, as the division process that the parasite undergoes within the bradyzoite formation creates more than two offspring from one mother cell (Sheffield, 2011).

1.4.1.3. Egress

For intracellular pathogens, egress is an essential phase of its life cycle. When there is no more space and nutrients within the host cell, the newly formed parasites lyse the parasitophorous vacuole and the host cell plasma membrane. This process occurs very quickly, and relies on potassium (K^+) and calcium (Ca^{2+}) signalling, ang gliding motility (Frénal & Soldati-Favre, 2009; Roiko & Carruthers, 2009).

Although egress is an active process, it is possible that it occurs without active motility of the parasite. It happens as a consequence of mechanical forces applied on the host cell plasma membrane as the volume of the parasitophorous vacuole increases (Frénal & Soldati-Favre, 2009).

1.5. Morphology and organelles

Toxoplasma exhibits a classic eukaryotic morphology, with easily recognizable organelles, unlike other *Apicomplexa* species like *Plasmodium* and *Theileria*, which have a reduced organization (Hager, Striepen, Tilney, & Roos, 1999).

T. gondii tachyzoite morphology, which is responsible for the acute phase of the infection caused by this parasite, is a high polarized cell, usually crescent shape, and is approximately the size of a red blood cell (2μm x 8μm) (Hill, Chirukandoth, & Dubey, 2005; de Souza, W., Belfort, 2014). The cell presents the nucleus in the rounded region, effectively dividing the cytoplasm into basal complex, located in the rounded posterior end, and apical complex, at the pointed anterior end. It has a pellicle (outer covering), several organelles including subpellicular microtubules, mitochondria, smooth and rough

endoplasmatic reticulum, Golgi apparatus, apicoplast, ribosomes, a micropore, and a well-defined nucleus (Hill et al., 2005).

The cytoskeleton of the parasite is split into three main structures: the apical complex, the inner membrane complex, and the basal complex. The apical complex is the feature that all *Apicomplexa* presents, as in their invasive stages the cells are always highly polarized (Håkansson, Morisaki, Heuser, & Sibley, 1999; Nicholas Jeremy Katris, 2017).

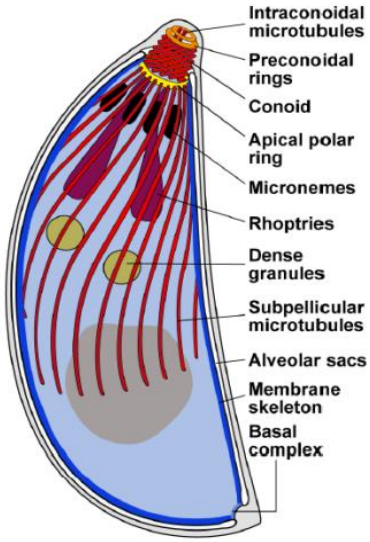

Figure 2 - Schematic representation of a *T. gondii* showing structural elements of the apical complex, the cell pellicle, and the secretory organelles (adapted from (Katris et al., 2014) with permission).

1.5.1. Apical Complex

Apicomplexan parasites share a group of cytoskeletal structures essential for parasite survival and pathogenesis of which the apical complex can be highlighted. Upon host cell invasion, the apical complex is extremely important as it offers both a semi-rigid framework and a focal point for secretory organelles, allowing them to release diverse invasion factors that mediate interaction with the host cell (Nicholas J. Katris et al., 2014).

Throughout Apicomplexa, the elements of the apical complex are highly conserved, including one or more polar rings, micronemes and rhoptries, conoid and sub-pellicular microtubules (Morrissette & Sibley, 2002; Gubbels & Duraisingh, 2012; de Souza, W., Belfort, 2014).

1.5.1.1. Conoid

The conoid is a small cone-shaped structure built around a core of tubulin that plays a mechanical role in invasion of host cells. This structure seems to act like a probe

during invasion and egress, and it is known that regulated secretion from this region provides essential factors for attachment, invasion and formation of the parasitophorous vacuole (Morrissette & Sibley, 2002; Hu et al., 2006; Skariah, Bednarczyk, McIntyre, Taylor, & Mordue, 2012).

1.5.1.2. Rhoptries and micronemes

Rhoptries and micronemes are secretory organelles that contain products needed for motility, adhesion to host cell, invasion of the host cell, and formation of the parasitophorous vacuole. The contents of these two structures is released, concomitant with a close interaction between the parasite and the host cell membranes (Morrissette & Sibley, 2002; Gubbels & Duraisingh, 2012).

1.5.1.3. Polar rings and sub-pellicular microtubules

As the pellicle of the parasite consists of two membranes: inner and outer membranes. The inner membrane terminates in a solid polar ring at the anterior end of the parasite, where the sub-pellicular microtubules are attached, and another polar ring at the posterior end. The sub-pellicular microtubules, which have their origin at the anterior polar ring, extend to the posterior ring and are believed to be associated with motility (Sheffield & Melton, 1968; Nichols & Chiappino, 1985).

1.6. Cellular Cytoskeleton

Many aspects of cell physiology are controlled by the cytoskeleton, including mitosis, cell division, cell polarity, etc. Moreover, the cytoskeleton receives, integrates, and transmits both intracellular and extracellular signalling cues. The cytoskeleton of eukaryotic cells is composed of three different types of fibers: actin filaments, intermediate filaments and microtubules (Mostowy & Cossart, 2012; Bezanilla et al., 2015).

The major component of the cytoskeleton is actin, an ATP-binding protein that co-exists in monomers (globular actin) and filaments (filamentous actin). These filaments are polar, and the two ends have distinct biochemical properties. For this reason, the fast-growing end is called the plus (+) end, and the slower-growing end is called minus (-) end. The polymerization of this filaments promoted ATP hydrolysis and the release of inorganic phosphate. (Welch & Mullins, 2002; Mostowy & Cossart, 2012).

The least stiff of cytoskeletal polymers are the intermediate filaments, as they resist tensile forces more effectively than compressive forces. Intermediate filaments are

non-polar due to the antiparallel orientation of the proteins assembled. They have the ability to crosslink either to other intermediate filaments, as well as to actin filaments and microtubules (Fletcher & Mullins, 2010; Mostowy & Cossart, 2012).

Microtubules are the most rigid of all intracellular cytoskeleton filaments, as their rigidity is a fundamental piece for all biological functions. Microtubules are cylindrical structures that are built from tubulin monomers bind to GTP molecules (Hawkins, Mirigian, Selcuk Yasar, & Ross, 2010; Mostowy & Cossart, 2012).

1.6.1. Tubulin heterodimer structure

Microtubules are filaments assembled from heterodimers of α-tubulin and β-tubulin into long polymers. The main difference between the two monomers resides in the guanine nucleotide binding. For α-tubulin, the bound to GTP is effectively sequestered, meaning it is non-exchangeable and not hydrolysed (N- site). On the other hand, β-tubulin bound to GTP is labile and exchangeable (E-site) (Nogales, Wolf, & Downing, 1998; Georgiev, 2003).

Although the monomer structure is very compact, it can be divided into three functional domains: the amino-terminal domain containing the nucleotide-binding region, an intermediate domain containing the Taxol-binding site, and the carboxy-terminal domain, which probably constitutes the binding surface for motor proteins (Nogales et al., 1998).

1.6.2. Microtubule cytoskeleton

The microtubule network is involved in several cellular phenomena, and for this reason there are two main sub-classes: stable and dynamic microtubules (Morrissette & Sibley, 2002b).

Dynamic microtubules are responsible for the organization of the cytoplasm, as well as the positioning of the nucleus and organelles. Meanwhile cellular division, a dynamic array of microtubules is responsible for segregation of chromosomes and orientation of the plane of cleavage. The ability to change between dividing and non-dividing stages is caused by dynamic instability in tubulin filaments, being able to stable growing and rapidly shrinking, allowing the microtubules cytoskeleton to reorganize rapidly (Desai & Mitchison, 1997; Fletcher & Mullins, 2010).

As for the stable microtubules, *T. gondii* cortical microtubules do not show dynamic instability and they are not depolymerized when free tubulin concentration is dramatically reduced (Francisco, 2020).

Dynamic instability means a profound nonequilibrium in which the ends of individual polymers transition randomly between periods of growth and shortening, requiring an energy source. As the only source of energy is GTP hydrolysis by β-tubulin during polymerization, it can be said that GTP hydrolysis powers dynamic instability (Desai & Mitchison, 1997; Goodson & Jonasson, 2020).

At the most fundamental level, polymerization dynamics allow the cytoskeleton to rapidly reorganize. In the polymerization phase, GTP-tubulin subunits are added to the end of a microtubule. After polymerization, the tubulin subunits hydrolyse their bound to GTP, releasing the hydrolysed phosphate. In the depolymerization phase, GDP-tubulin subunits are released from the microtubule ends very quickly (Desai & Mitchison, 1997).

1.6.3. Microtubule associated proteins (MAPs)

Beyond intrinsic dynamics (dynamic instability), there are proteins that can interact with microtubules regulating their dynamics. Microtubules assembled from pure tubulin heterodimers are an unstable structure, as it enables the microtubules to assemble quickly to respond to changes in the cellular and exterior environment, and as an influence of regulatory proteins. To fulfil its diverse functions, microtubules assemble into distinct arrays that are characterized by a defined architecture and dynamics. The formation of these arrays requires specialized proteins that regulate its assembly – MAPs (microtubule associated proteins). MAPs can be functionally categorized as stabilizers, destabilizers, capping proteins, and bundlers/cross-linkers (Bodakuntla, Jijumon, Villablanca, Gonzalez-Billault, & Janke, 2019; Goodson & Jonasson, 2020)

1.6.4. Tubulin folding pathway

The maturation of tubulin heterodimers is a complex multistep process that involves the interactions of tubulin subunits with molecular chaperones and tubulin cofactors (TBCA-E). These proteins are also strong candidates to regulate the microtubules dynamics, as they regulate the tubulin pool to polymerize (Francisco, 2020).

The cytosolic chaperonin containing T-complex polypeptide 1 (CCT complex) mediates the folding pathway, requiring additional presence of GTP, a set of protein cofactors and native tubulin. Upon its interaction with CCT, tubulins follow two different pathways in which α-tubulin is captured by cofactor B (TBCB) and β-tubulin is captured by cofactor A (TBCA). Cofactors E (TBCE) and D (TBCD) intervene and capture respectively α- and β-tubulin, forming a super complex. Then, cofactor C (TBCC) interacts with this complex, promoting GTP hydrolysis and releasing the α-/β-tubulin-GDP heterodimers. However, in order to become competent to polymerize into microtubules, the exchange of GDP by GTP is needed (Tian et al., 1997; Gonçalves, Tavares, Carvalhal, & Soares, 2010; Francisco, 2020).

1.6.5. Tubulin folding cofactors

More than being required for proper tubulin folding and heterodimer formation, tubulin cofactors have also shown to participate in tubulin dissociation, transitory tubulin storage and tubulin degradation processes, suggesting this proteins might be the key to understand how microtubules dynamics occurs. Plus, being αβ-tubulin heterodimer a very stable protein, its dissociation might be provided by these cofactors (M. Lopez-Fanarraga et al., 2007; Kortazar et al., 2007).

1.6.6. Tubulin cofactor A (TBCA)

Tubulin cofactor A binds to β-tubulin released from CCT, being then transferred to TBCD. TBCA seems to function as a β-tubulin chaperone, capturing and storing β-tubulin monomers after dissociation reaction, working as a reservoir of partially folded β-tubulin subunits (Tian et al., 1996; Melki, Rommelaere, Leguy, Vandekerckhove, & Ampe, 1996).

1.6.7. Tubulin cofactor C (TBCC)

Tubulin cofactor C its required for the formation of tubulin heterodimers, as it promotes the assembly of both α-tubulin and β-tubulin peptides. This cofactor is necessary to complete the folding process, forming a super complex (with TBCD, α-tubulin, β-tubulin and TBCE) and releasing the αβ-tubulin heterodimers after GTP

hydrolysis cycles (Hage-Sleiman, Herveau, Matera, Laurier, & Dumontet, 2010; Garcia-mayoral et al., 2011).

1.6.8. Tubulin cofactor D (TBCD)

Tubulin cofactor D is a post-CCT chaperone that binds to GTP-bound β-tubulin released from TBCA, being part of the super complex formed by TBCD/ β-tubulin/TBCE/α-tubulin (Tian et al., 1996; Martín, Fanarraga, Aloria, & Zabala, 2000)

1.6.9. Tubulin cofactor E (TBCE)

Tubulin cofactor E binds to α-tubulin after it is released from TBCB. Plus, TBCE is able to act as part of a GTP-activating complex on native tubulin and to sequester α-tubulin subunits from native heterodimers, suggesting a dual role of this cofactor in the biogenesis and degradation of the tubulin heterodimer (Tian et al., 1996; Kortazar et al., 2007).

1.6.10. Tubulin cofactor B (TBCB)

Tubulin cofactor B is a post-CCT chaperone that plays a role in α-tubulin folding and degradation pathways thought molecular mechanisms essential for controlling tubulin dynamics, and consequently microtubules dynamics. This cofactor plays an important role, as it is involved in a plethora of human disorders, such as: cancer (Vadlamudi et al., 2005), neurodevelopmental malformations (Tian et al., 2010), schizophrenia (Martins-de-Souza et al., 2009), and neurodegenerative processes (W. Wang, Ding, & Allen, 2005).

The αβ-tubulin heterodimer dissociation process only occurs in the presence of both TBCB and TBCE. When α-tubulin monomer is released from the tubulin heterodimer, it is stabilized within a complex formed by TBCB and TBCE. The disassembly of this ternary complex results in either TBCB/ α-tubulin and free TBCE, or TBCE/ α-tubulin and free TBCB. The complex formation between TBCB and TBCE enhances the efficiency of TBCB to dissociate tubulin *in vivo* and *in vitro* (Carranza et al., 2013; Serna et al., 2015).

Cofactor B is theoretically a protein of ~30 kDa, but it can be observed as having different molecular mass values. Under gel filtration analysis TBCB behaves as a ~130

kDa protein, by mass spectrometry has ~27 kDa and by SDS-PAGE it corresponds to a single polypeptide of 38 kDa (Tian et al., 1997).

Despite all we know about TBCB, its function *in vivo* is not yet completely understood. For example, in *Schizosaccharomyces pombe*, a knockout of TBCB results in a decrease of α-tubulin levels that correlates with an affected microtubules network and defects it all its actions (Radcliffe & Toda, 2000). On the other hand, in mammalian cells TBCB knockdown does not affect tubulin levels nor the microtubules network (Vadlamudi et al., 2005). However, the overexpression of TBCB induces microtubule depolymerization in human cells through its interaction with TBCE (Kortazar et al., 2007).

For mammalian cells, TBCB is a soluble cytoplasmatic protein in interphase cells, even though it is often located at the centrosome and at the base of primary cilium. However, during mitosis, TBCB can also be spotted around spindle microtubules and with the progression of cell division it concentrates on the midbody microtubules and progressively disappears from the centrosome. These locations suggest that TBCB can bind to microtubules (Carranza et al., 2013).

In *T. gondii* TBCB presents a polarized distribution as it is located mainly at the anterior region of the parasite, close to the apical complex and immediately below the conoid. In addition, it can also be spotted just below the apical region and at the posterior pole. Recently, studies performed by Francisco and colleagues with the objective to identify its location in the cellular division process demonstrated that TBCB does not seem to be present at the mitotic spindle, being barely detected in the forming daughter cells. However, the cytoplasmatic location of the protein was confirmed, possibly interacting with the sub-pellicular microtubules (Francisco, 2020).

Plus, with overexpression of this cofactor in *T. gondii* it is possible to observe a reduction in the invasion rate of the parasite within the host cell. Although, it shows no differences in replication and egress assays (Francisco, 2020).

Tubulin C-terminal ends are usually exposed to the outer surface of the microtubules, providing binding sites for several MAPs and molecular motors (Z. Wang & Sheetz, 2000; Meyho & Laka, 2005). They are also subjected to post-transcriptional modifications (PTMs) after their polymerization into microtubules, modifying the microtubules' stability and dynamics by giving them different biochemical and biophysical properties Cortical microtubules can be modulated by PTMs, and there are studies that confirm the presence of proteins along their length (Hu, Roos, & Murray, 2002). Our group are considering as an hypothesis for TBCB to be a partner of these proteins and

its overexpression may generate an imbalance in there interactions, affecting consequently the microtubules' organization and host cell invasion (Francisco, 2020).

That being said, TBCB proves to be an essential protein to the parasite, being required to generate a functional microtubules network that is needed for invasion, replication and cell polarity processes (Francisco, 2020).

The main goal of this project was to characterize the TBCB interactome in *T. gondii* to determine its mechanism of action. For that, we used the BioID technique, which uses proximity dependent biotin identification of proteins.

Identification of protein-protein associations is a fundamental approach to the study of protein function. BioID is a method that allows to screen for candidate protein interactions based on proximity-dependent cellular biotinylation using a promiscuous bacterial biotin ligase fused to a bait protein. The biotinylated proteins can easily be captured using conventional methods (as immunoprecipitation) and identified using mass-spectrometry. Based on its mechanism of action, this technique is effective when applied to insoluble and membrane-associated proteins, as these two classes of proteins are usually refractory to screening with conventional approaches. Plus, it has ability to identify weak and transient interactions, to screen for interactions in a relatively natural cellular setting, and temporal inducibility of biotin labelling. Moreover, non-interacting but neighbouring proteins are also labelled, giving important information on the proteomic landscape surrounding the target protein in native environments (Roux, Kim, & Burke, 2013; Kim et al., 2016)

1.7. Objectives

The main goal of our work was to produce *T. gondii* transgenic strains expressing BirA in fusion with TBCB (BirA_TBCB) and only BirA (acting as control).

Concretely, we aim to:

- Perform the molecular cloning in *T. gondii* expression vectors;
- Transfect *T. gondii* with the plasmid constructs;
- Select and isolate *T. gondii* clones, by limit dilutions;
- Confirm correct recombinant protein expression and biotinylation capability;
- Optimize the biotinylation and pull-down conditions.

2. Materials and Methods

2.1. Nucleic acid molecular biology

2.1.1. Bacterial strains, growth media and culture conditions

In this study it had been used an *Escherichia coli* strain for general cloning. This strain is DH5α, a versatile strain used for general cloning and sub-cloning applications.

E. coli cells were cultured at 37ºC in two different ways: in liquid medium, with vigorous shaking (~200 rpm), and in solid medium.

The liquid culture medium used was LB (NZYtech, Lisbon, Portugal) and the solid medium used was LB agar (Nzytech). For plasmid selection, according to the plasmid resistance, the media were supplemented with ampicillin (100 μg/mL). These cells were stored for a few days in solid medium, at 4ºC.

2.1.2. Transformation of competent cells by heat shock

One aliquot (100 μL) of competent cells was thawed on ice. Half of the final volume of the ligation reaction product was added to the bacteria suspension and incubated on ice for 20 minutes. After that, the cells were subjected to heat shock by placing the tubes in a 42ºC water bath for 90 seconds and putting the tubes back on ice for about 1 minute. Then 500μL of LB medium was added to the cells, and these were incubated for 30 minutes at 37ºC, in a shaking incubator (~200 rpm). Finally, the tubes were centrifuged at 13000 rpm for 2 minutes, 400μL of the supernatant were discarded, the pellet was resuspended, and the cells were plated on LB agar supplemented with ampicillin.

2.1.3. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique used to amplify DNA sequences. This method involves using short DNA sequences called primers to select the portion of the genome to be amplified, allowing it to be studied in detail.

2.1.3.1. DNA Polymerases

When downstream cloning was required, the Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used since the correct DNA sequence of the PCR product was crucial. This polymerase's processivity-enhancing domain results in shorter

extension times, more robust and high yield amplification, and the ability to extend long templates in a fraction of the time.

The DreamTaq™ Green DNA Polymerase (Thermo Fisher Scientific) was used for regular PCRs.

2.1.4. Colony PCR

After a molecular cloning experiment, colony PCR is a convenient high-throughput method for determining the presence or absence of the desired insert in plasmid constructions, directly from bacterial colonies.

A single bacteria colony that has grown up following the transformation step was picked to a PCR tube with 10 μL of purified water. For each colony we picked, it was made a replica on a LB agar plate. We took 1 μL from this solution to a new PCR tube, where the PCR reaction was going to take place. The replica plate was incubated at 37ºC overnight, and then stored at 4ºC.

A PCR Master Mix with the DreamTaq™ Green DNA Polymerase (Thermo Fisher Scientific), with specific oligonucleotides to the insert, was prepared and add to the PCR tubes. Amplification reaction was prepared using 10x DreamTaq™ Green Buffer, 10 mM dNTPs (Thermo Scientific), forward and reverse primers (Table 1) at a final concentration of 30 μM each, 25 mM magnesium cloride and 0.2 U/μl of enzyme.

PCRs with a final volume of 10 μl were processed with an initial denaturation at 95ºC for three minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at the primer pair specific temperature for 30 seconds, extension at 72ºC during 30 seconds per 1000 bp of the expected amplicon, and a final extension at 72ºC for one minutes.

The PCR products were analysed by DNA electrophoresis. Positive colonies were inoculated in liquid LB medium overnight at 37ºC, in a shaking incubator (~200 rpm), for plasmid purification.

2.1.5. Agarose gel electrophoresis

Gel electrophoresis is a technique used to separate charged molecules.

The DNA analysis was done using agarose gels at a concentration of 1% (w/v) in 1X Tris-acetate-EDTA (TAE) buffer, supplemented with the DNA-binding dye GreenSafe

Premium (Nzytech). 6X DNA loading dye was added to the DNA samples in a proportion of 1:5 and the electrophoresis was performed in TAE at a constant voltage of 100V. The GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) was run with the samples to estimate the weight of the DNA fragments.

After that, the gel was visualized on ChemiDoc XRS+ System (BioRad).

2.1.6. Extraction of plasmid DNA from *E. coli*

The plasmid purification from *E. coli* was made using two different commercial kits for plasmid extraction and purification (CANVAX; QIAGEN), accordingly to the manufacturer's instructions.

The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica membrane in the presence of high concentrations of salt.

2.1.7. Determination of nucleic acid concentration and purity

NanoDrop 2000c spectrometer (Thermo Scientific) was used to quantify and determine the purity of the nucleic acids.

2.1.8. DNA Sequencing

DNA sequencing was performed by Eurofins Genomics through Nzytech.

2.2. Molecular cloning of TBCB BioID vectors

Molecular cloning to obtain the TBCB BioID vectors was done using the In-Fusion HD Cloning kit (Takara Bio), according to the manufacturer's instructions. All vectors and oligonucleotides used in this work are listed in Tables 1 and 2.

The BirA and BirA-TBCB sequences were first amplified from the *DD_myc_ BirA_TBCB* vector.

Molecular cloning to obtain the TBCB BioID vectors was done by a ligation independent cloning technique using the In-Fusion HD Cloning kit (Takara Bio), according to the manufacturer's instructions. All vectors and oligonucleotides used in this work are listed in Tables 1 and 2.

The final vectors, designated pmorn_BirA and pmorn_BirA_TBCB will be transfected into *T. gondii* RH tachyzoites (see section 2.4) to generate the pmorn_BirA and pmorn_BirA_TBCB strains, respectively.

The ***DD_myc_ BirA_TBCB*** vector is built in the Tub8-DD-myc-GFP-MyoAtail-HX plasmid backbone, a kind gift from Markus Meissner (Wellcome Centre for Molecular Parasitology, University of Glasgow, Glasgow, Scotland, UK. And Department of Veterinary Sciences, Ludwig-Maximilians-Universität, Munich, Germany).

The **BirA** sequence included in vector *DD_myc_ BirA_TBCB* was originally obtained from the *pcDNA5-Flag-BirA* vector, provided by Helena Soares (Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa and Centro de Química Estrutural–Faculdade de Ciências da Universidade de Lisboa).

The TBCB sequence corresponds to the *T. gondii* TBCB cDNA previously cloned by our group (Francisco S, 2020).

The *T. gondii* MORN1 is a protein constitutively expressed at moderate levels, with known sub-cellular localization in T gondii (Gubbels *et al*. 2006). Its promoter, pmorn1, will be used to drive expression of BirA and BirA-TBCB. For that purpose, plasmid *pmorn1-Morn1-Myc-Cat* (a kind gift from Marc-Jan Gubbels, Department of Biology, Boston College, USA) will be used for In-Fusion cloning of BirA and BirA-TBCB sequences. The *pmorn1-Morn1-Myc-Cat* vector (previously hydrolized with the *Bgl*II and *Asc*I restriction endonucleases, which simultaneously remove the *Morn1-Myc* fragment and linearize the plasmid) was used for ligation at vector:insert molar ratios of 1:2 and 1:8 in a final 10μL volume. In all ligations were used 50 ng of plasmid DNA. The reaction was incubated for 15 minutes at 50ºC, and then put into ice. Whenever needed storage was at -20ºC.

The final vectors, designated pmorn_BirA and pmornBirA_TBCB were transfected into T. gondii RH tachyzoites to generate the TgBirA and TgBirA_TBCB strains, respectively.

Table 1 – Oligonucleotides sequences used in this work.

Oligonucleotides used for cloning	
Oligonucleotide name	**Oligonucleotide sequence**
BirA_forward	5' ATGAAGGACAACACCGTGCCC 3'
BirA_reverse	5' CGCGCCTGTACAGATATCTTAA 3'
TBCB_reverse	5' GCTGGACGAAATCTAATTAATTAAT 3'
pmornBirA_forward	5' ACCGTTGTCCACCAGATCATGAAGGACAACACCGTGCCC 3'
pmornBirA_reverse	5' CGGGCAGCTTCTGGCGCGTTAAGATATCTGTACAGGCGCG 3'
pmornTBCB_reverse	5' CGGGCAGCTTCTGGCGCGATTAATTAATTAGATTTCGTCCAG 3'
Oligonucleotides used for colony screening and sequencing	
pmorn_F	5' GTATCTCCTGTCTTGAATTA 3'
pmorn_R	5' GAACGAAAGCGAGTTGC 3'

2.2.1. DNA amplification of BirA and BirA_TBCB fragments

The BirA and BirA_TBCB sequences were amplified using as a template the *DD_myc_BirA_TBCB* vector with the high-fidelity enzyme Phusion High Fidelity DNA Polymerase (Thermo Scientific).

Amplification reactions were prepared using 5x Phusion HF Buffer, 10 mM dNTPs (Thermo Scientific), forward and reverse primers at a final concentration of 10 μM each and 0.2 U/μl of enzyme.

PCRs with a final volume of 25-50 μl were processed with an initial denaturation at 98ºC for 2 minutes, 35 cycles of denaturation at 98ºC for 10 seconds, annealing at 63ºC for 30 seconds, extension at 72ºC during 30 seconds per 1000 bp of the expected amplicon, and a final extension at 72ºC for three minutes.

2.3. Generating BirA and BirA_TBCB *T. gondii* expressing strains for BioID analysis

2.3.1. Mammalian cell lines and culture

The cell lines used were Human foreskin fibroblasts (HFF) cells. HFF cells can be cryopreserved in culture medium with 20% Fetal Bovine Serum (FBS) heat inactivated, and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen for many years. After thawing, HFF cells should not be sub-cultured for more than 30 passages, as they lose effectiveness. HFF confluent monolayers were used to culture and maintain *T. gondii* tachyzoites.

This cell lines are adherent cells and were maintained in subconfluent monolayers through passages with Trypsin-EDTA (0.05 %) phenol red (Invitrogen) treatment, to detach them from the flask surface. They were grown in Dulbecco's Modified Eagle's Medium (DMEM), high glucose, GlutaMAX™ (Invitrogen), supplemented with 10 % FBS heat inactivated (Invitrogen), without any antibiotics, in a 5 % CO_2 humidified atmosphere at 37º C.

2.3.2. *Toxoplasma gondii* strain and culture

In this study, we used *T. gondii* type I strain RHΔ*Hx* referred here as RH (Donald, Carter, Ullman, & Roos, 1996).

Being *T. gondii* an obligate intracellular parasite, its tachyzoites were maintained in HFF cells cultured in DMEM + GlutaMax™ (Thermo Fisher Scientific) medium, supplemented with 10% FBS heat inactivated, in a 5% CO_2 humidified atmosphere at 37ºC.

Before it can lyse the cells, *T. gondii* undergoes many rounds of replication inside the cell. Once they manage to lyse the cells, they became extracellular and need to be transferred to new cells.

All strains were provided by Markus Meissner (Wellcome Centre for Molecular Parasitology, University of Glasgow, Glasgow, Scotland, UK. And Department of Veterinary Sciences, Ludwig-Maximilians-Universität, Munich, Germany).

2.3.3. *T. gondii* cryopreservation

The way to cryopreserve intracellular parasites as *T. gondii* is freezing them while they are still within the host cells.

For long-term storage, *T. gondii* parasites were frozen within HFF host cells. The day after most HFF cells presented large parasites vacuoles. The host cells were treated with 0.4 ml Trypsin-EDTA (0,05%) phenol red (Thermo Fisher Scientific) to detach them from the flask surface.

The freezing media was made with 20% FBS heat inactivated, 10% DMSO and 70% DMEM + GlutaMAX™ media (Thermo Fisher Scientific). After being detached from the surface, the cells were centrifuged, and the pellet was resuspended in 1 ml of freezing media and transferred to a cryotube. The cryotubes were placed overnight at -80ºC in a freezing container (Mr. Frosty, Thermo Fisher®) so it can slowly decrease 1ºC/minute. The next day, cryotubes were transferred to liquid nitrogen storage.

2.3.4. DNA precipitation

DNA precipitation is usually performed to concentrate DNA and/or purify nucleic acids from salts or buffers. For the purification of DNA fragments from the plasmid extraction product it was performed ethanol precipitation.

To each DNA solution was added 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of cold 100% ethanol (-20ºC). The mixture was incubated overnight at -20ºC. After that, the mixture was centrifuged at 13,000 rpm for 1 hour at 4ºC. The supernatant was removed and was added 1 mL of cold 70% ethanol (-20ºC). The mixture was then centrifuged at 13,000 g for 15 min at 4ºC. The supernatant was removed, and the pellet was air dried under sterile conditions.

2.3.5. Transfection of *T. gondii*

The transfection of *T. gondii* was done by electroporation. The Nucleofector 2b Device (Lonza), was used with the Amaxa Basic Parasite Nucleofector Kit 2 (Lonza) and the program U-033.

For *T. gondii* transfection, 10-15 μg of plasmid DNA were precipitated, air-dried in the laminar flow hood, and resuspended in the transfection solution provived with the Amaxa Basic Parasite Nucleofector Kit 2 (Lonza).

The DNA was precipitated and dissolved in 100 μl of electroporation buffer from the kit. Following, the fresh parasites were centrifuged at 800 G for 10 minutes at 4ºC, the supernatant was discarded, and the pellet was washed twice with PBS 1x. The sediment was resuspended in 100 μ of electroporation buffer containing the DNA.

After electroporation, the parasites were added to a T25 flask with confluent HFF cells to maintain the parasite culture.

2.3.6. *T. gondii* cloning selection

After transfection, the parasites were added to a 25cm^3 cell culture flask. Once they lysed completely the cells, they were passed to another T25 flask with confluent HFF cells containing 20 nM of chloramphenicol.

2.3.7. *T. gondii* cloning by limiting dilutions

After selection, the isolation of a clonal stable line of parasites can be done by limiting dilutions. With this aim, serial dilutions of the transfected parasite pool were inoculated in a 96 well plate containing confluent HFF cells. Consequently, some wells received only one parasite. After that, wells that presented only one lysis plaque indicated a parasite clone. The parasites within this well were then isolated and transferred to a 24 well plate with confluent HFF cells.

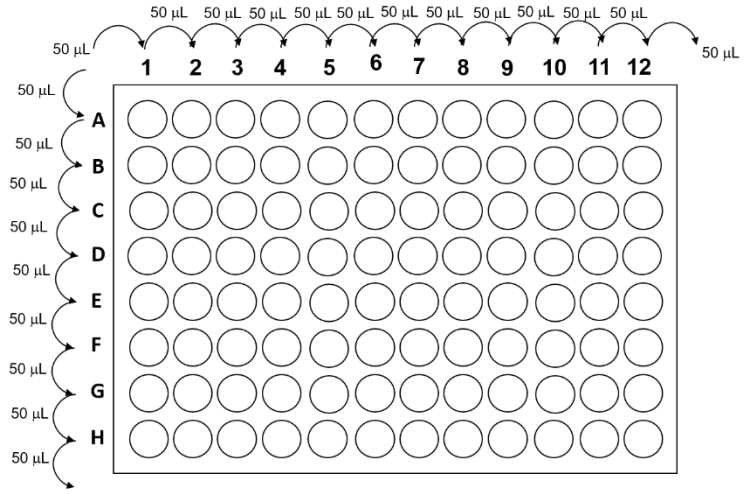

Figure 3 – Schematic representation of the limit dilutions performed.

2.4. Protein assays

2.4.1. Preparation of parasite protein extracts for SDS-PAGE

Two different protocols were used to prepare protein extracts from parasites for SDS-PAGE. The first protocol used H Solution [250 mM sucrose (Sigma-Aldrich), 100 mM NaCl (Merck), 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich) pH 7.6, 2 mM ethylenediaminetetraacetic acid (EDTA, Bio-Rad), 0.1% (v/v) Triton X-100 (Sigma-Aldrich), 1x Halt Protease and Phosphatase Inhibitor Cocktail EDTA-free (Thermo Scientific)], and the second protocol used RIPA buffer (25 mM Tris · HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxucholate, 0.1% SDS) (Thermo Scientific), as the last one usually shows better results in solubilizing proteins.

For each sample, parasites were spindown in 1,5 mL tubes at 800 g for 10 minutes at room temperature. The pellet was washed twice with PBS 1x (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM $KH2PO_4$). The final pellet was lysed with 100 µL of H Solution (50 mM Hepes pH 7.6, 2 mM EDTA, 100 mM NaCl, 250 mM Sucrose, and 0.1% Triton X-100) with Protease and Phosphatase Inhibitor Cocktail, EDTA-free 1x (Thermo Scientific), and the protein extracts were centrifuged at 17000 g for 20 minutes at room temperature. The supernatant was transferred to a new tube. The same protocol was followed for RIPA buffer (Thermo Scientific) with Protease and Phosphatase Inhibitor Cocktail, EDTA-free 1x (Thermo Scientific).

For the soluble fraction of the protein extract, the quantification was done by the Bradford Protein Assay.

2.4.2. Determination of protein concentration by Bradford Protein Assay

The Bradford Protein Assay is used to measure the concentration of total protein within a sample. As the sample is added to Coomassie blue G-250 assay reagent, it binds to proteins through ionic interactions between sulfonic acid groups and positive protein amine groups through Van der Waals attractions (Tal, Silberstein, & Nusser, 1985). The resultant blue colour is measured at 595nm following a short incubation at room temperature.

200 μL of Bradford reagent (Bio-Rad) was added to a new tube, with 800 μL of H_2O and 1 μL of protein soluble extraction. The solutions were mixed by the inversion of the tubes and incubated for 15 minutes to 1 hour at room temperature. Finally, the absorbance was measured at 595 nm and the sample concentration was calculated using a protein standard curve (y=0,0814x [y: absorbance; x: concentration]).

2.4.6. SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is an analytical technique that allows to separate proteins based on their molecular weight. When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. In SDS-PAGE, it eliminates the influence of the structure and charge, and proteins are separated solely based on polypeptide chain weight.

We used 5% stacking gels and 12% resolution gels. SDS-PAGE was carried out with the Mini-Protean Tetra Vertical Electrophoresis Cell (BioRad) and the protein ladder was the NZYColour Protein Marker II (Nzytech). For each gel, 10 μL of protein ladder was loaded. The gel migrates in running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at 25 mA/gel.

For the insoluble fraction (pellet), it was first resuspended in 25 μL of urea 8 M and 25 μL of loading buffer 2X. The samples were then boiled for 5 minutes and loaded into the well.

2.4.7. Transfer of proteins from SDS-PAGE to nitrocellulose membrane

After SDS-PAGE, proteins were transferred into a nitrocellulose membrane. It was used a Wet/Tank blotting system from BioRad. The 3mm filter papers, the gel, the membrane and the sponges were all embedded in transfer buffer (25 mM Tris base, 192 mM glycine, 10% methanol in water) for a couple minutes. The sandwich for blotting was put together and the blot was run either at 300 mA for 75 minutes or at 30 V overnight.

2.4.8. Ponceau S Staining

Ponceau S staining is a rapid and reversible staining method used for the detection of protein bands on nitrocellulose membranes, among others. Ponceau S is a negative stain that binds to the positively charged functional groups of the protein (amino group) and the non-polar regions of the protein. This technique is useful to locate and identify the proteins transferred by electrophoresis into nitrocellulose membranes before the antibody-mediated detection.

2.4.9. Immunoblotting

Immunoblotting is a technique that allows analysis of proteins via antigen-antibody specific reactions. Membranes were washed with PBS to remove the Ponceau-S staining and then were blocked in 5% Molico skimmed milk powder (Nestlé) in PBS 1x at 4ºC overnight. After blocking, the membrane was incubated for 1 hour with the primary antibody (see table 3) in an orbital shaker. The membrane was then washed 3 times with PBS 0.1% Tween for 10 minutes. After that, the membrane was incubated with the secondary antibody (labelled with HRP) (see table 3) for 1 hour in an orbital shaker. It was then washed 3 times with PBS 1x for 10 minutes. HRP conjugated secondary antibodies were detected with the Amersham ECL Prime Blocking Reagent, according to manufacturer's instructions. The visualization was done using the ChemiDoc XRS+ System (BioRad).

Table 2 – List of antibodies used

Antibodies			
Antibody	**Produced in**	**Dilution**	**Source**
Polyclonal anti-*T. gondii* TBCB	Rabbit	1:1000 1:500	In house production
Polyclonal anti-BirA	Rabbit	1:1000	Invitrogen
Peroxidase AffiniPure Anti-Rabbit	Goat	1:2000 1:1000	Jackson ImmunoResearch Europe Ltd

2.4.10. Biotinylation assays and detection

BioID is a unique method to screen for physiologically relevant protein interactions that occur in living cells. This technique harnesses a promiscuous biotin ligase to biotinylate proteins based on proximity. The ligase is fused to a protein of interest and expressed in cells, where it biotinylates proximal endogenous proteins (Roux, Kim, Burke, & May, 2018).

Freshly egressed $2x10^7$ TgBirA and TgBirA_TBCB tachyzoites were inoculated onto HFF confluent cells in T25 flasks with 150 μM biotin in DMEM + GlutaMAX™ (Thermo Scientific) with 1% FBS. T25 flasks were incubated for three days. At this point, tachyzoites had fully lysed the host cell monolayer and the culture medium was harvested to recover the parasites. The resulting pellets were washed twice with PBS 1x and centrifuged at 800 g for 10 minutes to obtain medium free parasites pellets. These were prepared for protein electrophoresis and precipitation.

To detect the biotinylation, we used immunoblotting with streptavidin HRP. Membranes were incubated with blocking solution [PBS 2.5% (w/v) BSA (NZYTech), 0.4% (v/v) Triton X-100 (Sigma-Aldrich)] overnight at 4 ºC. After blocking, membranes were probed with Streptavidin-HRP (Thermo Fisher Scientific), diluted in blocking solution (1:50.000), during one hour with orbital shaking at room temperature, followed by three wash steps with PBS 10 minutes. HRP was detected with ChemiDoc XRS+ System (BioRad).

2.5. Bioinformatics

The DNA confirmation was done using BLAST (Basic Local Alignment Search Tool) tool of databases like NCBI (http://www.ncbi.nlm.nih.gov/).

Multiple Alignment of the DNA sequences were done using CLC Sequence Viewer 8.

3. Results

3.1. Establishing the TBCB BioID analysis system in *T. gondii*: construction of the recombinant plasmids for transfection

We will set up a TBCB BioID analysis system by constructing two *T. gondii* transgenic strains: a BirA control strain, expressing the BirA protein and a BirA_TBCB test strain, expressing the BirA protein in fusion to the TBCB protein from T. gondii.

In the first section the BirA and BirA_TBCB sequences are amplified from plasmid *DD_myc_BirA_TBCB* (v9 backbone) and cloned into plasmid *pmorn1_Morn1_Myc_Cat* (v9 backbone) to generate the vectors pmorn1_BirA and pmorn1_BirA_TBCB.

In a second section, *T gondii* tachyzoites will be transfected and isolated by limit dilution to generate TgBirA and TgBirA_TBCB clonal lines.

T. gondii clonal lines will be analysed by immunoblotting to assure correct expression of the BirA and BirA-TBCB recombinant proteins by testing the biotinylation levels.

3.1.1. Amplification of BirA and BirA_TBCB sequences for recombination

The first step we took in our approach was to clone BirA and BirA_TBCB sequences using PCR with specific primers. The sequences were amplified from *DD_myc_BirA_TBCB* (v9 backbone) vector. To perform a PCR we used specific primers. In this case, we used primers BirA_forward and BirA_reverse (Table 1) to amplify BirA sequence, and primers BirA_forward and TBCB_reverse (Table 1) to amplify the whole BirA_TBCB sequence. To analyse PCR products, we performed an 1% agarose gel electrophoresis (Figure 3).

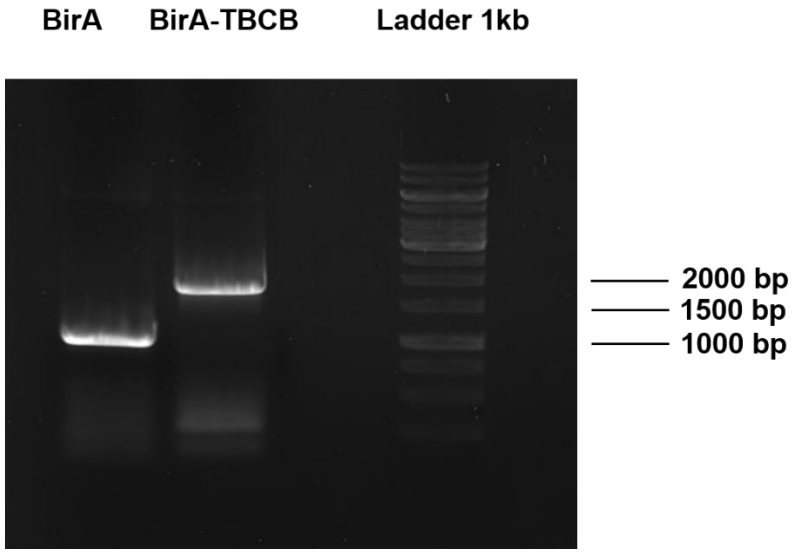

Figure 4 – Amplification of BirA and BirA_TBCB fragments from vector *DD_myc_BirA_TBCB*. Amplification of BirA and BirA_TBCB sequences from vector *DD_myc_BirA_TBCB* generated fragments with the expected molecular weights. **BirA**: sequence of BirA amplified with specific primers BirA_forward and BirA_reverse generates a fragment of 1008 bp. **BirA_TBCB:** sequence of BirA_TBCB amplified with specific primers BirA_forward and BirA_TBCB_reverse generates a fragment of 1833 bp. **Ladder**: Gene Ruler 1kb DNA Ladder (Thermo Scientific).

BirA is an enzyme that can catalyse the covalent attachment of biotin to proteins that it interacts with (Y. Li & Sousa, 2012). BirA coding sequence has 1008bp. When in fusion with TBCB, it shows 1833 bp. Upon analysis of the results obtained, we can easily observe that we obtained one obvious band around 1000bp for BirA and another obvious band a bit lower than 2000bp. This information is consistent with the reference lengths we have for BirA and BirA_TBCB, respectively.

Once the correct amplification of BirA and BirA_TBCB fragments based on fragment length was confirmed, these were purified from the gel with the agarose gel purification kit (QIAGEN), quantified by Nanodrop analysis and were both used for subsequent recombination with vector *pmorn1_Morn1_Myc_Cat* using the In-Fusion technique.

To prepare the plasmid for bacteria transformation, we needed a recombination step that allowed us to insert our sequence of interest (BirA and BirA_TBCB) into *pmorn1_Morn1_Myc_Cat* (v9 backbone) plasmid. This step allowed to get the plasmid with the coding sequence for BirA and BirA_TBCB with a moderate promotor - *pmorn1*. To perform the recombination, we used the In-Fusion method to incorporate BirA and BirA_TBCB sequences, to generate the corresponding vectors. As so, we used pmornBirA_forward and pmornBirA_reverse (Table 1) to incorporate BirA sequence into

the plasmid, and pmornBirA_forward and pmornTBCB_reverse (Table 1) to generate a BirA_TBCB vector.

3.2. Generating pmorn1_BirA and pmorn1_BirA_TBCB vectors

Upon recombination, the plasmid was incorporated into *E. coli* by heat shock technique and the cells were plated in LB medium + 1 μL ampicillin/mL. The plasmid itself has ampicillin resistance gene, which means it can survive in a medium with ampicillin. The cells that have incorporated correctly the plasmid would survive in a medium with ampicillin, whereas the cells that did not correctly incorporated the plasmid would not survive. After an overnight incubation at 37ºC with shaking, positive colonies were collected and analysed by Colony PCR. Colony PCR is a method for rapidly screening colonies for bacteria that have grown up on selective media following a transformation step (Bergkessel & Guthrie, 2013).

Twelve different grown colonies were obtained either for BirA and BirA_TBCB. We subjected these colonies to a Colony PCR technique, using specific primers - pmorn_F and pmorn_R (Table 1).

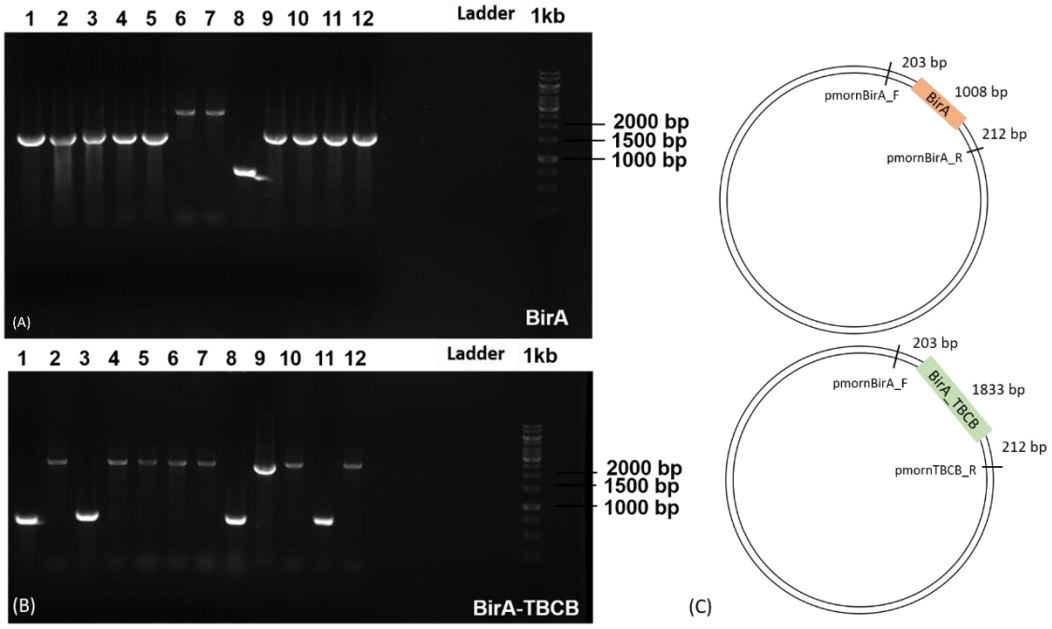

Figure 5 – Confirmation of pmorn1_BirA and pmorn1_BirA_TBCB vectors. (A) BirA: Colony PCR was made using primers pmornBirA_forward and pmornBirA_reverse, with an expected length of 1423 bp. **(B) BirA_TBCB:** Colony PCR was made using pmornBirA_forward and pmornTBCB_reverse, with an expected length of 2248 bp. **Ladder:** Gene Ruler 1kb DNA Ladder (Thermo Scientific). **(C)** Shows a graphic representation of the cloning plasmids. The **lines** are identified and represent the primers used.

If the recombination process did not work and bacteria were transformed with a closed plasmid without any DNA insert, we were expecting to see bands ~400 bp, as the region amplified by our primers is 415 bp length. These primers hybridize 203 bp upstream BirA sequence, and 212 bp downstream BirA or TBCB sequence, for pmornBirA and pmornBirA_TBCB plasmids respectively and, for this reason, if the insert is not there, it is expected to see only the empty plasmid. In Figure 4A, in colony 9, and in Figure 3B, in colonies 1, 3, 8 and 11, the bacteria were transformed with a closed plasmid.

On the other hand, the sequence amplified by the primers when BirA and BirA_TBCB were incorporated upon recombination are 1423 bp and 2248 bp, respectively. Based on this information, we can easily identify nine positive colonies for the cells transformed with BirA plasmid (Figure 4A) and only one positive colony for cells with BirA_TBCB plasmid (Figure 4B).

The work proceeded using colony 2 for BirA and colony 9 for BirA_TBCB. We extracted the plasmid DNA from these *E. coli* specific colonies using commercial kits for plasmid extraction.

3.3. Sequencing BirA and BirA_TBCB fragments to confirm integrity

The extraction products were sent to Eurofins Genomics through Nzytech for sequencing. The sequenced product of BirA and BirA_TBCB fragments were aligned with BirA and BirA_TBCB sequences from database respectively, using CLC Sequence Viewer 8 and the comparison between the sequences we obtained and the ones from database was done using BLAST (Basic Local Alignment Search Tool) from NCBI (http://www.ncbi.nlm.nih.gov/).

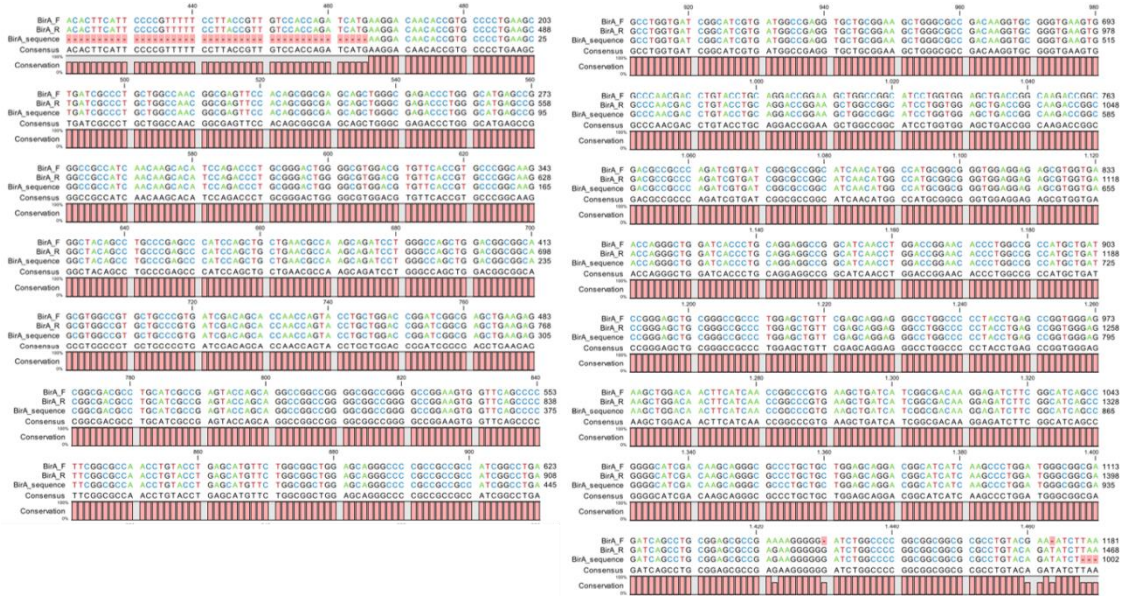

Figure 6 – Alignment of sequences obtained with BirA_forward and BirA_reverse primers. The multiple sequence alignment of BirA sequence was performed using CLC Sequence Viewer 8, and the final output was given with NCBI Nucleotide BLAST Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Gaps are denoted with hyphens. According to NCBI Nucleotide BLAST Tool, it has 100% identity.

We used NCBI Nucleotide BLAST Tool to align the sequences obtained with BirA_forward and BirA_reverse primers, and we obtained a 100% nucleotide identity for each fragment, meaning the plasmid we obtained has the BirA sequence we predicted.

Figure 7 – Alignment of sequences obtained with BirA_forward and TBCB_reverse primers. The multiple sequence alignment of BirA_TBCB sequence was performed using CLC Sequence Viewer 8, and the final output was given with NCBI Nucleotide BLAST Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Gaps are denoted with hyphens. According to NCBI Nucleotide BLAST Tool, it has 100% identity. (A) Alignment of BirA_TBCB sequence obtained with prime forward. (B) Alignment of BirA_TBCB sequence obtained with prime reverse.

We used NCBI Nucleotide BLAST Tool to align the sequences we obtain for BirA_TBCB with forward and reverse primers. We obtained a 100% nucleotide identity,

which means the sequence we obtained in the plasmid was the same as the BirA_TBCB sequence we predicted.

Once we confirmed the sequences obtained were correct, we proceeded for *T. gondii* transfection, using the Nucleofector 2b Device (Lonza), with the appropriate kit.

3.4. Selection and isolation of *T. gondii* clonal lines TgBirA and TgBirA_TBCB strains

After transfection, *T. gondii* strains were exposed for 2 weeks to medium with 20 nM chloramphenicol. To isolate the clones, we did limited dilutions for each one of the final strains of *T. gondii* (TgBirA and TgBirA_TBCB). So, from the pool we obtained after transfection, we added 50 μl to the first well, and transferred 50 μl from the first well to the second (both horizontally and vertically), discarding 50 μl from the last wells. To select which clones we were using, we observed the 96 well plate at inverted optical microscopy and searched for well that presented only one lysis plaque. It means that ideally only one parasite was dropped on that well upon limited dilutions, and all the parasites that followed it were clones originated from only one parasite. Either for TgBirA and TgBirA_TBCB we identified 10 individual parasites presenting only one lysis plaque each one, being 1A8, 1B9, 1H2, 2A8 and 2F3 for TgBirA, and 1C5, 1C6, 1C7, 2B6 and 2B7 for TgBirA_TBCB (Figure 7A and 7B). These clones were maintained in either 24 well plates or T25 flasks, for analysis.

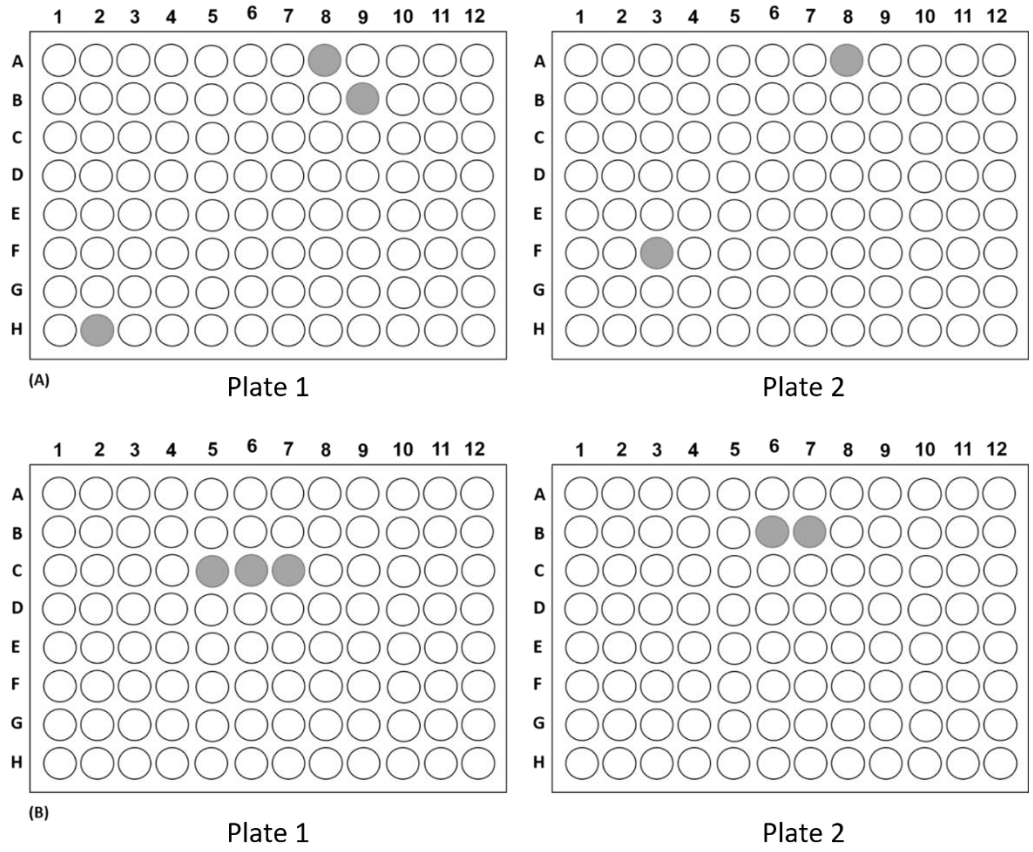

Figure 8 – Positive wells we obtained upon selection and isolation of the *T. gondii* clones. (A) We identified 5 wells (1A8; 1B9; 1H2; 2A8; and 2F3) as having only one lysis plaque, selecting those clones for further investigation. (B) We identified 5 wells (1C5; 1C6; 1C7; 2B6; and 2B7) as having only one lysis plaque, selecting those clones for further investigation.

3.5. Verification of TBCB protein expression

We decided to use SDS-PAGE followed by Western Blot with a polyclonal antibody against TBCB protein (Table 2) to verify if the recombinant protein was being expressed upon apparently correct sequence. We started by preparing soluble and insoluble protein extracts from the three strains (TgRH, TgBirA and TgBirA_TBCB) using H Solution.

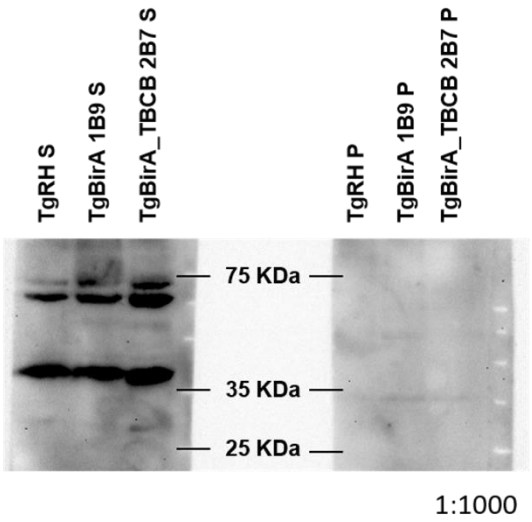

Figure 9 – Verification of presence of TBCB protein. Soluble and insoluble protein fractions was extracted from the three strains of *T. gondii* (Tg_RH, TgBirA and TgBirA_TBCB). TgRH and TgBirA were being used as negative controls for the presence of overexpressed TBCB protein, as they are expected to express only endogenous TBCB. S stands for soluble fraction, as P stands for pellet. We can observe the results from the Western blot technique using 1:1000 primary antibody against TBCB with 1:2000 secondary antibody.

Endogenous TBCB is a polypeptide of 38 kDa and it is expected to be able to see endogenous TBCB in the three strains, as it is normally produced by the cells (Lopez-Fanarraga et al., 2007). At first, we decided to use the primary polyclonal antibody against TBCB at 1:1000 dilution with a secondary antibody labelled with HRP at 1:2000. Consequently, we can observe bands around 35 kDa on the three strains (Figure 8), which might be the endogenous TBCB protein. In contrast, overexpressed TBCB, because it is fused with BirA, has a total weights of 73 kDa. In Figure 8 we can also observe a band around 75 kDa, but this band is also present in both TgRH and TgBirA, the negative controls. This band, which might have been justified by the presence of overexpressed TBCB if only occurred in TgBirA_TBCB, indicates that we are probably in the presence of an unspecific band.

3.6. Verification of BirA protein expression

We also decided to use Western blot techniques to confirm the presence of BirA protein in TgBirA and TgBirA_TBCB clones, using a polyclonal antibody against BirA protein (Table 2). At first, in the same way that we did for previous Western blot analysis,

we extracted proteins from the three strains (TgRH, TgBirA and TgBirA_TBCB) using H solution protocol (Figure 9).

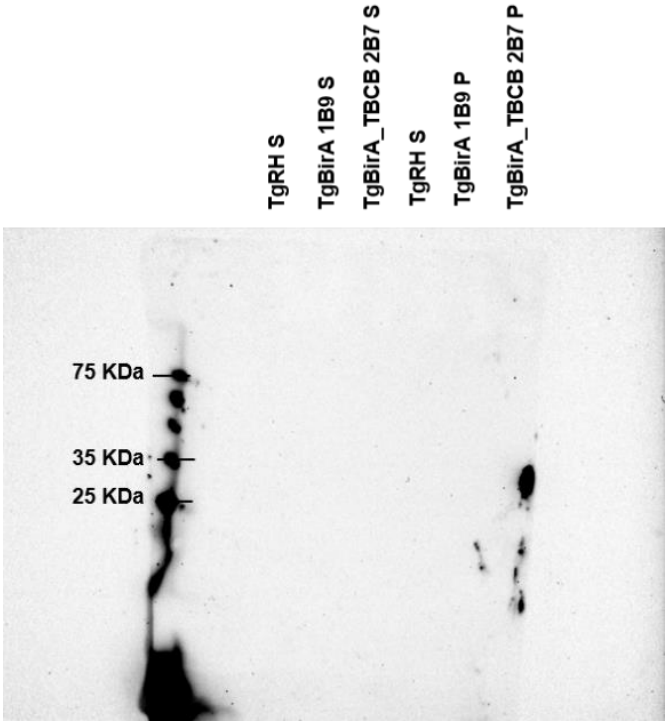

Figure 10 – Verification of presence of BirA protein. We started by preparing soluble and insoluble protein extracts from the three strains of *T. gondii* (Tg_RH, TgBirA and TgBirA_TBCB) using H Solution. TgRH is being used as negative controls for the presence of BirA protein. S stands for soluble fraction, as P stands for pellet. We can observe the results from the Western blot technique using 1:1000 primary antibody against BirA with 1:1000 secondary antibody. The same amount of protein was used for each well upon previous quantification analysis on Nanodrop.

BirA is a protein of 35 kDa (Y. Li & Sousa, 2012), and we were expecting to see bands of ~35 kDa for TgBirA and TgBirA_TBCB, and none for TgRH as this strain is acting as a negative control. We used a polyclonal antibody against BirA protein (Table 2) at 1:1000 and a secondary antibody labelled with HRP (Table 2) at 1:1000, according to manufacturers' recommendations. However, we did not observe any bands. We repeated this technique several times, changing antibody concentration and exposure times, all of them with no success. We do not have a clear explanation for these results, the antibody against BirA protein was new, recently bought but we did not have any positive control to test its integrity and we cannot exclude that its viability might have been affected.

3.7. Biotin assay

Despite our previous results, we considered the non-detection of overexpressed TBCB and BirA proteins as a problem with the antibodies used, so we decided to test if there was biotinylation activity. We used Streptavidin, a protein that has an high affinity for biotin to perform an Western blot.

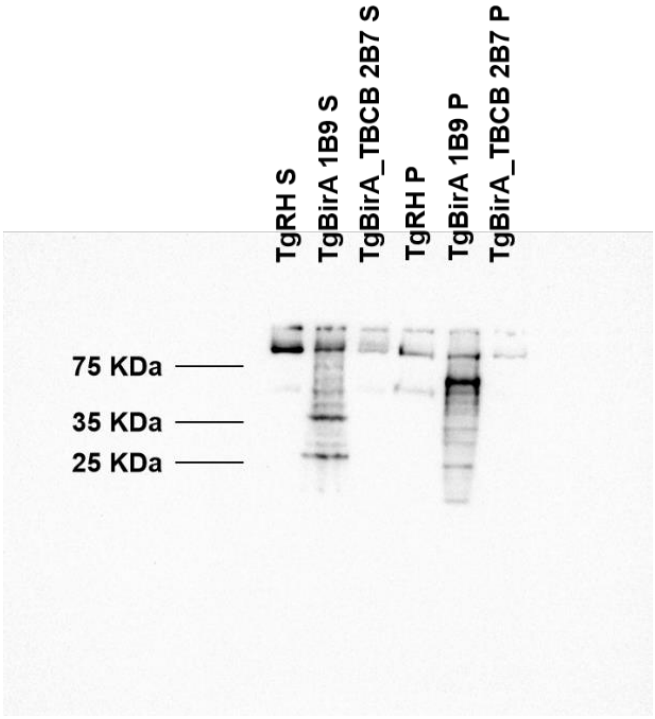

Figure 11 – Evaluation of biotinylation levels in *T. gondii*. Protein was extracted from the three strains of *T. gondii*. TgRH is being used as a negative control. S stands for soluble fraction of the protein extract, and P stands for pellet. The same amount of protein was used for each well upon previous quantification analysis on Nanodrop.

In a first approach, we decided to test the same clones we started our work with, being TgRH, TgBirA 1B9 and TgBirA_TBCB 2B7. At first, we subjected the clones to a medium with 150 μM biotin for two days We performed SDS-PAGE followed by Western blot, using 1:1000 HRP Streptavidin (Thermo Scientific). Upon analysis of the results obtained (Figure 10) it was possible to conclude that TgBirA clone was working as expected, showing biotinylation levels around 35 kDa, suggesting BirA related biotinylation. However, the biotinylation levels we observed for TgBirA_TBCB were the same as in TgRH.

After this, we decided to test all TgBirA_TBCB clones to search for a working clone. We subjected the TgBirA_TBCB strains, together with a TgBirA clone (acting as a positive control) and TgRH (acting as a negative control), to medium with 150 μM biotin

for a couple days. After that, we extracted proteins from the three strains using H Solution protocol and did SDS-PAGE followed by Western blot technique, using 1:1000 HRP Streptavidin (Thermo Scientific).

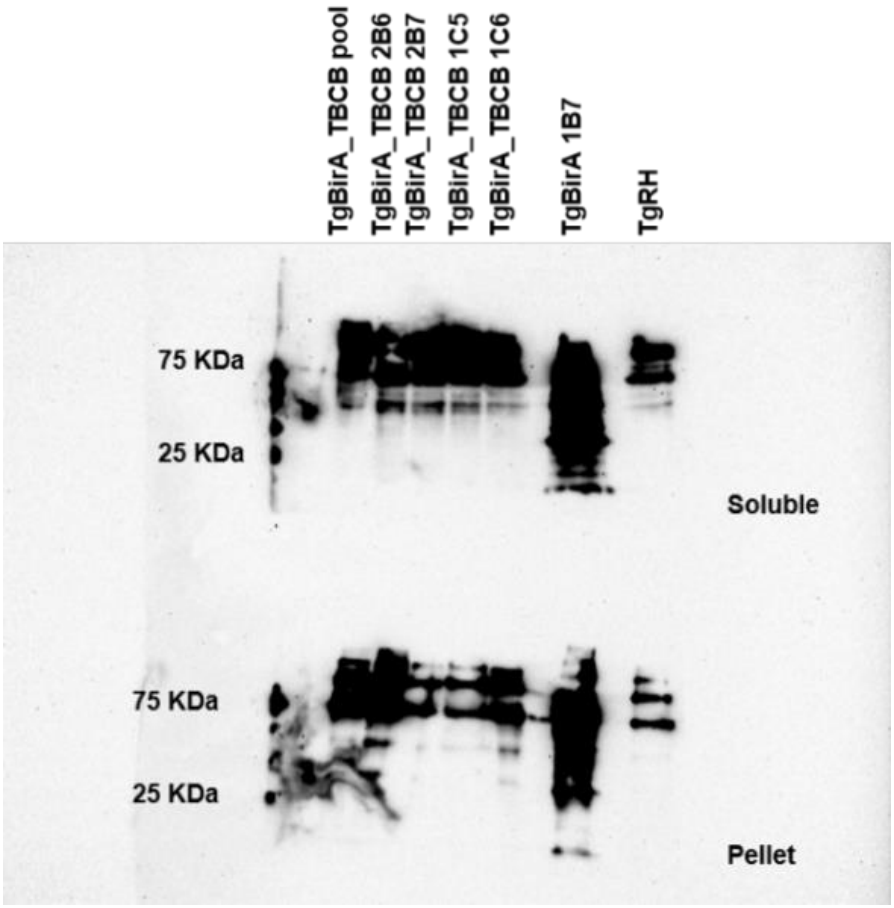

Figure 12 – Testing all BirA_TBCB clones to evaluate biotinylating levels. Protein was extracted from the three strains of *T. gondii* using H. solution protocol. TgRH is being used as a negative control. TgBirA is being used as a positive control. The same amount of protein was used for each well upon previous quantification analysis on Nanodrop.

From the results we obtained (Figure 11), we could see positive bands for two clones: TgBirA_TBCB 2B6 and TgBirA_TBCB 1C6. However, the biotinylation levels demonstrated by these clones were not as strong as the one observed in TgBirA. So, we decided to test all TgBirA clones in order to evaluate if there was any clone that had weaker activity. Again, we exposed all TgBirA clones to a medium with 150 μM biotin for a couple days. After that, we extracted proteins from the three strains using H Solution protocol followed by SDS-PAGE and Western blot using 1:1000 HRP Streptavidin (Thermo Scientific).

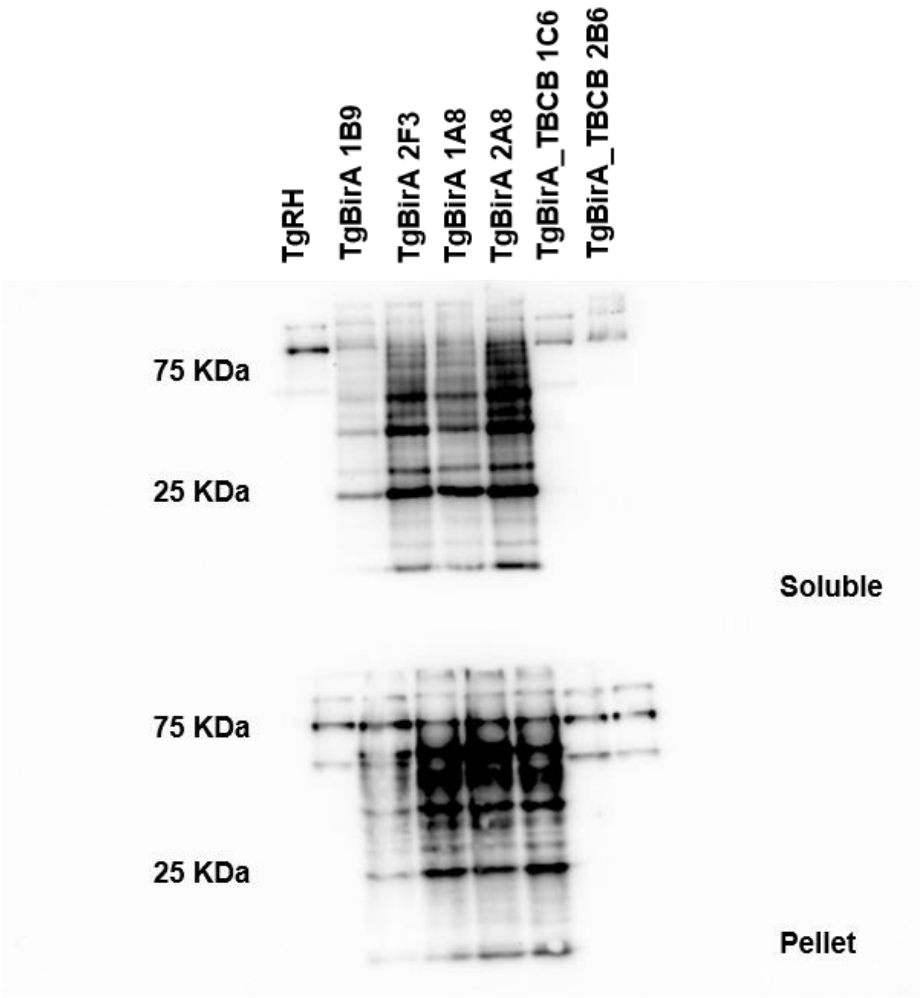

Figure 13 – BirA clones analysis to evaluate biotinylating levels. Protein was extracted from the three strains of *T. gondii* using H. solution protocol. TgRH is being used as a negative control. TgBirA_TBCB is being used as a reference for biotinylation levels. The same amount of protein was used for each well upon previous quantification analysis on Nanodrop.

As can be seen in Figure 12, it was possible to identify TgBirA 1B9 as having the weaker levels of biotinylation, even though all TgBirA clones were working. At the same time, we tested TgBirA_TBCB 2B6 and TgBirA_TBCB 1C6, serving as a reference for biotinylation levels, even though we were not able to see biotinylation different from the obtained in TgRH.

At this point, we had selected the weakest biotinylating TgBirA functional clone and the possible TgBirA_TBCB functional clone for further analysis. We also maintained TgRH to be used as a negative control. We decided to repeat the test using these selected clones. We exposed them to a medium with 150 μM biotin for a couple days. After that, we extracted proteins from the three strains using RIPA buffer (Thermo Fisher

Scientific), we sonicate the protein extracts with the aim of solubilizing the proteins of interest, and performed SDS-PAGE followed by Western blot using 1:1000 HRP Streptavidin (Thermo Scientific).

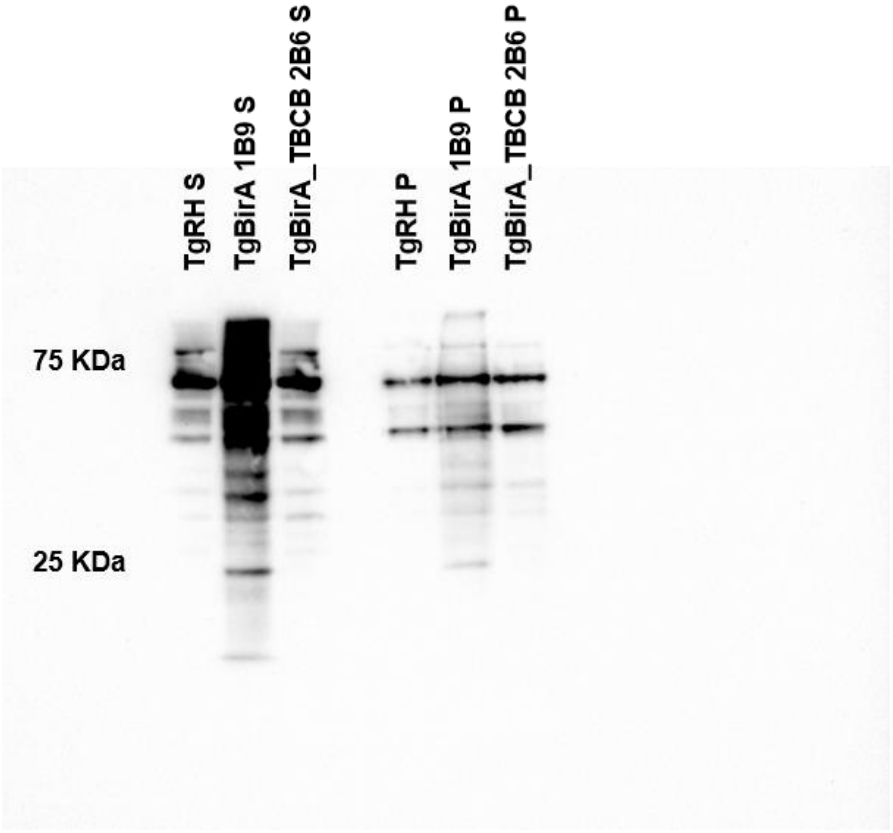

Figure 14 – Comparison of biotinylation levels in TgBirA 1B9 and TgBirA_TBCB 2B6 clones. Protein was extracted from three strains of *T. gondii* using RIPA solution. TgRH is being used as a negative control. S stands for soluble fraction of protein extract, and P stands for pellet. The same amount of protein was used for each well upon previous quantification analysis on Nanodrop.

The results we obtained (Figure 13) showed the expected biotinylation levels for TgRH (negative control) and for TgBirA, so we can conclude that TgBirA 1B9 is a functional clone that can be used for further work. However, we did not obtain favourable results for TgBirA_TBCB clones, as their biotinylation levels were no different from TgRH.

4. Discussion

The main goal of this project was to characterize TBCB interactome using BioID, and for that the present thesis contributed by producing *T. gondii* transgenic strains expressing BirA in fusion with TBCB (BirA_TBCB) and only BirA (as a control). To achieve this goal, we cloned *T. gondii* expression vectors pmornBirA and pmornBirA_TBCB, transfected *T. gondii* with the plasmid constructs, isolated and tested *T. gondii* clones.

The first step was to clone BirA and BirA_TBCB sequences using PCR with specific primers. The sequences were amplified from *DD_myc_BirA_TBCB* (v9 backbone) vector. To perform PCR we used specific primers and Phusion High-Fidelity DNA Polymerase (Thermo Scientific).

To prepare the plasmid for bacteria transformation, a recombination step was needed as it allowed us to insert our sequence of interest (BirA and BirA_TBCB) into *pmorn1_Morn1_Myc_Cat* (v9 backbone) plasmid.

The method we used for this was In-Fusion HD Cloning Kit (Takara Bio), which is a recombination method different from the classic one that involves restriction enzymes to prepare the plasmids and DNA (cDNA) fragments and also requires the presence of a ligase. This method is usually used when, for example, the complete sequence of the plasmids we are manipulating is not known, or which restriction enzymes should be used. Briefly, this method allows a fast, directional cloning of one or more fragments of DNA simultaneously into any vector in a single reaction. It also does not need restriction digestion, phosphatase treatment, or ligation.

Upon bacteria transformation, we obtained grown colonies for each plasmid, and we subjected them to a Colony PCR using specific primers (Figure 3). We continued our work extracting plasmid DNA from colony 2 for BirA and colony 9 for BirA_TBCB, and sent the product to Eurofins Genomics through Nzytech.

From the results we obtained for BirA sequence alignment (Figure 4), we can say that our sequence presents 100% nucleotide identity to the sequence that we were expecting to match. It means that the sequence we obtained from amplification of BirA sequence from *DD_Myc_BirA_TBCB* plasmid was the same as the one we predicted. Results obtained for BirA_TBCB sequence alignment (Figure 5) showed also 100% nucleotide identity when compared to the predicted sequence, which means that the sequence we obtained was the same as the one we expected.

When performing a cloning procedure, there are two paths we can follow to obtain the fragments: 1) hydrolysis from a plasmid followed by separation through electrophoresis and purification from the gel, or 2) using PCR technique to clone the fragment, preferentially using a proof-reading polymerase.

As mentioned before, we used Phusion Phusion High-Fidelity DNA Polymerase (Thermo Scientific) as it is a high-fidelity polymerase and provides more robust amplifications, to amplify BirA and BirA_TBCB sequences from the original plasmid *DD_Myc_BirA_TBCB*. As we can see from the results obtained with the sequencing, we can say that our goal for the use of this polymerase was achieved, as the sequences we obtained where the same as we expected, with 0% error.

Once the sequences were confirmed, we transfected *T. gondii* parasites with BirA and BirA_TBCB plasmids by electroporation. With the aim of selecting transfected *T. gondii*, we kept the parasites in a T25 flask with confluent HFF cells containing 20 nM of chloramphenicol. After nearly two weeks, only the parasites that have incorporated the plasmid were able to survive, as the resistance gene for chloramphenicol was inserted in the plasmid.

The next step was to isolate a clonal stable line of parasites. For this, we used limit dilutions. In this case, limit dilutions allow to generate a monoclonal cell line resulting in cell populations that are more likely to retain stable transgene expression. We obtained 5 clonal lines for each one of the strains, so we needed to test them.

We used SDS-PAGE followed by Western Blot with a polyclonal antibody against TBCB protein (1:1000 primary antibody and 1:2000 secondary antibody labelled with HRP). to verify if the proteins were being produced as expected. The same amount of protein was loaded into each well, upon quantification analysis by Nanodrop. Endogenous TBCB is a protein of 38 kDa and we were expecting to be able to see it along the three strains, as it is a protein normally produced by the cell. We obtained bands (~35 kDa) for the three strains, that correspond to endogenous TBCB. However, TBCB when in fusion with BirA is a protein of 73 kDa. We did obtain bands ~75 kDa on the three strains, as we were expecting to see only in TgBirA_TBCB. It was important to analyse both soluble and pellet fractions, as TBCB protein might be associated with cellular structures that are not lysed with the lysis buffer, becoming part of the pellet fraction.

In addition, we also tested TgBirA clones using SDS-PAGE followed by Western blot with a polyclonal antibody against BirA protein to confirm its presence. The same amount of protein was loaded into each well, upon quantification analysis by Nanodrop. Even though the antibody was new, we did not obtain any bands.

We were not able to see the expected proteins with the antibodies and methods we used, its presence in the cell could not be discarded. We used BioID, which allows the detection of protein-protein interactions that occur in intact cells and involves the expression of a protein of interest (TBCB) fused to a modified version of a bacterial biotin ligase (BirA). BirA adds an ATP to biotin to produce a highly reactive biotinoyl-5'-AMP intermediate, which subsequently reacts with a lysine in the target point. At this time, the protein is biotinylated (Baudouin, Pfeiffer, & Ochsenreiter, 2020).

We chose BioID against other methods, as immunoprecipitation and yest two-hybrid system. The reason was, in the first place, and as mentioned before, BioID allows to detect interactions within an intact cell and it can detect transient protein interactions, unlike immunoprecipitation where the detection is made *in vitro* and does not allow to detect transient interactions. Additionally, with BioID it is possible to identify proteins in the surroundings of our bait, including insoluble and membrane-associated proteins, on the contrary of yeast two-hybrid system, in which the cells used are not the original cell from where the protein belong, and its interaction must occur in the nucleus of the cell, otherwise it may not produce a positive interaction. BioID allows the biotinylation of proteins in the proximity of our protein of interest, together with the proteins in direct interaction with it. This can be seen as a disadvantage, as it is easy to get lost within all the proteins that might be in the proximity, but can also be seen as a strong point, as we can identify proteins that interact within those pathways (Stephens & Banting, 2000; Brückner, Polge, Lentze, Auerbach, & Schlattner, 2009; Sears, May, & Roux, 2019; Peipei, Meng, Li, & Li-jun, 2019; Baudouin et al., 2020).

Despite the results we obtained before, we decided to evaluate the biotinylation levels on the clones. In our first approach, we test the same clones we started our work with (gRH, TgBirA 1B9 and TgBirA_TBCB 2B7) (Figure 9). We performed SDS-PAGE followed by Western blot using HRP Spreptavidin. The same amount of protein was loaded into each well, upon quantification analysis by Nanodrop. The results obtained showed biotinylation levels suggesting BirA related biotinylation for TgBirA clone, and no biotinylation different from the negative control for TgBirA_TBCB.

We then decided to test all TgBirA_TBCB clones to search for a functional clone. We applied the same method, using the same amount of protein to load each well, and clones TgBirA_TBCB 2B6 and TgBirA_TBCB 1C6 showed some different biotinylation levels from the negative control.

So, we tested all TgBirA clones in order to identify the clone that presented the least strong biotinylation levels, so we could compare with TgBirA_TBCB clone. The same amount of protein was loaded into each well. It was possible to identify TgBirA 1B9 as having the weaker levels of biotinylation, even though all TgBirA clones were working.

At this point, we compared the biotinylation levels between the strongest TgBirA_TBCB clone and the weakest TgBirA clone. The amount of protein loaded into the wells was the same for each sample, upon quantification analysis by Nanodrop. We also decided to use RIPA lysis buffer instead of H Solution that we have been using until this point. The reason behind it is because RIPA buffer allows to strongly solubilize proteins, which means more protein will be collected within the soluble fraction, and TBCB protein can be transferred to this fraction, as we have mainly identified biotinylating activity in the pellet fraction. Even though we were able to obtain more protein extract on the soluble fraction, having more intense bands, we were still not able to see any differences between TgBirA_TBCB and the negative control.

5. Conclusion

The main goal of our work was to produce *T. gondii* transgenic strains expressing BirA in fusion with TBCB (BirA_TBCB) and only BirA (acting as a control).

The cloning of the expressing vectors and the transfection of *T. gondii* with our constructs was done successfully. Plus, it was possible to select and isolate *T. gondii* clones, obtaining a functional TgBirA clone and a non-functional TgBirA_TBCB clone.

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