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Toxoplasma ubiquitin-like protease 1, a key enzyme in sumoylation and desumoylation pathways, is under the control of non-coding RNAs

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ABSTRACT

Sumoylation and desumoylation are reversible pathways responsible for modification of protein structures and functions by the reversible covalent attachment of a small ubiquitin-like modifier (SUMO) peptide. These pathways are important for a wide range of cellular processes and require a steady supply of SUMO, which is generated by an enzymatic reaction catalysed by the ubiquitin-like protease (Ulp) family. Here we show by functional complementation analysis that the Ulp1 of *Toxoplasma gondii* (*Tg*Ulp1) can rescue a growth-deficient phenotype of a yeast-*Ulp1* knockout. Recombinant *Tg*Ulp1 is an active enzyme capable of removing SUMO from a sumoylated substrate. Using a clonal transgenic strain of *T. gondii* expressing an epitope-tagged version of *Tg*Ulp1, we detected that the expression of *TgUlp1* is modulated by *Tg*-miR-60, the most abundant species of micro RNA found in the *T. gondii* type 1 strain. The introduction of *Tg*-miR-60 inhibitor caused an increase in *TgUlp1* expression and its enzymatic activity, as well as affecting the parasite's growth fitness. Moreover, we discovered a polyadenylated antisense RNA transcribed from the *TgUlp1* locus, referred to as *TgUlp1*-NAT1 (*TgUlp1*-natural antisense transcript 1). Both *Tg*-miR-60 and *TgUlp1*-NAT1 confer a regulatory function by down-regulating the expression of *TgUlp1* and affecting the sumoylation and desumoylation pathways in *T. gondii*.

1. Introduction

Toxoplasma gondii, a pathogenic parasite of the phylum Apicomplexa, is the cause of toxoplasmosis in birds and mammals. Being the single member of the *Toxoplasma* genus, *T. gondii* is known for its capability to infect any warm-blooded mammal. In humans, toxoplasmosis is found in every country, with sero-positivity rates ranging from <10% to over 90% (Pappas et al., 2009). A clinical severity of toxoplasmosis is found in immunocompromised individuals and in congenital infections (Furtado et al., 2011). The life cycle of *T. gondii* consists of two stages; a sexual reproduction stage in the gut of its feline definitive hosts, and an asexual reproduction stage in any of its hosts. The asexual stage contains two distinct forms; a rapidly growing form called a tachyzoite, and a latent cyst-encapsulated form called a bradyzoite. In the tachyzoite form, replication of the that form is limited by a healthy immune response. However, the conversion from the tachyzoite form to the bradyzoite form allows the parasite to evade the host's immune response. In the bradyzoite form, the parasite remains dormant but retains its ability to reconvert to the rapidly growing tachyzoite form. When the host's immune system becomes weakened, the reconversion could initiate a recurring infection in the infected host.

To maintain its infective ability in addition to its survival, *T. gondii*, similar to other eukaryotes, relies on highly coordinated mechanisms of transcriptional, post-transcriptional and post-translational gene regulation. Among these mechanisms, the covalent modifications of proteins are considered energetically favourable mechanisms to allow for rapid and reversible alteration of protein functions (Hay, 2005). Phosphorylation/dephosphorylation and acetylation/deacetylation of proteins can induce structural changes and alter the activities and localizations of proteins. Similarly, the formation and removal of an iso-peptide bond between the ε-amino group of an internal lysine residue in proteins and the C-terminal carboxyl group of ubiquitin-like (Ubl) proteins has been shown to increase the proteins' stability and affect their functions (Johnson, 2004; Hay, 2005).

Small ubiquitin-like modifiers (SUMO, ~100 amino acids (aa)) belong to the Ubl family. While sharing only 18% amino acid sequence identity with ubiquitin (~80 aa), SUMO adopts a tertiary structure of the C-terminal Ubl domain superimposable on that of ubiquitin. SUMO is highly conserved in all eukaryotes. While only one gene is encoded for SUMO in lower eukaryotes including

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T. gondii and other Apicomplexans (Braun et al., 2009; Reiter et al., 2013), multiple SUMO genes are expressed in plants and mammals (Johnson, 2004; Cappadocia and Lima, 2018). These SUMO genes encode SUMO precursors, which carry a short peptide blocking their C-terminus. The removal of the C-terminal amino acid residues of the SUMO precursor is carried out by enzymes of the ubiquitin-like protease (Ulp) family to expose a C-terminal diglycine motif that is required for conjugation (Fig. 1A). The mature form of SUMO undergoes a stepwise reaction that is catalysed by an E1 activating enzyme, E2 conjugating enzyme and E3 ligase. These enzymatic reactions are unique to the sumoylation pathway (Cappadocia and Lima, 2018). Sumoylated proteins then become substrates of Ulp, which hydrolyzes the iso-peptide bond between SUMO and the protein. This reaction makes the post-translational modification reversible and provides free SUMO to be used for the conjugation reactions of other proteins (Fig. 1A).

Sumoylation and desumoylation pathways are highly conserved in nearly all eukaryotes, and essential for the regulation of several vital cellular processes including transcription regulation and chromatin remodelling, DNA repair and cell cycle progression (Liu and Shuai, 2008; Hickey et al., 2012; Everett et al., 2013; Hendriks and Vertegaal, 2016). A comprehensive compilation of human SUMO proteomics showed that proteins involved in pre-mRNA splicing, the ribosome and ribosome biogenesis formed the largest cluster of sumoylated proteins (Hendriks and Vertegaal, 2016). Notably, sumoylation often targets a protein group, rather than an individual protein (Psakhye and Jentsch, 2012). In *T. gondii*, a genetic manipulation to abolish the expression of a SUMO-encoded gene resulted in a lethal phenotype (Braun et al., 2009). The overexpression of SUMO also gave rise to a lethal phenotype. The expression of recombinant SUMO was successful only when the coding sequence was placed under the control of its cognate promoter. Indirectly, these findings suggest the importance of the sumoylation pathway in *T. gondii*. Moreover, the study of SUMO proteomics in *T. gondii* identified over 100 sumoylated proteins that are involved in translation, metabolism and post-translational modification, and protein degradation (Braun et al., 2009). The study also indicated that sumoylation could play a role in host cell invasion and cyst genesis (Braun et al., 2009).

In this study, we focus on a *T. gondii* homolog of Ulp, named *Tg*Ulp1, whose expression can complement the function of *Saccharomyces cerevisiae* Ulp1 (*Sc*Ulp1). Most importantly, our study indicated that *TgUlp1* is regulated by non-coding RNA (ncRNA), namely micro RNA (miRNA) and antisense RNA. As an emerging class of regulatory biomolecules, ncRNAs make up a significant portion of transcripts of eukaryotic organisms and play an important role in many biological processes such as cell development and viability (Dykes and Emanueli, 2017). However, their mechanisms and functions are still under investigation. Here we provide experimental evidence that *TgUlp1* is a bona fide target of *Tg*-miR-60, an abundant miRNA species in the *T. gondii* type I strain (Braun et al., 2010). *TgUlp1* expression is negatively regulated by *Tg*-miR-60, whose action could be countered by a *Tg*-miR-60 inhibitor. The introduction of a *Tg*-miR-60 inhibitor resulted in an increase in

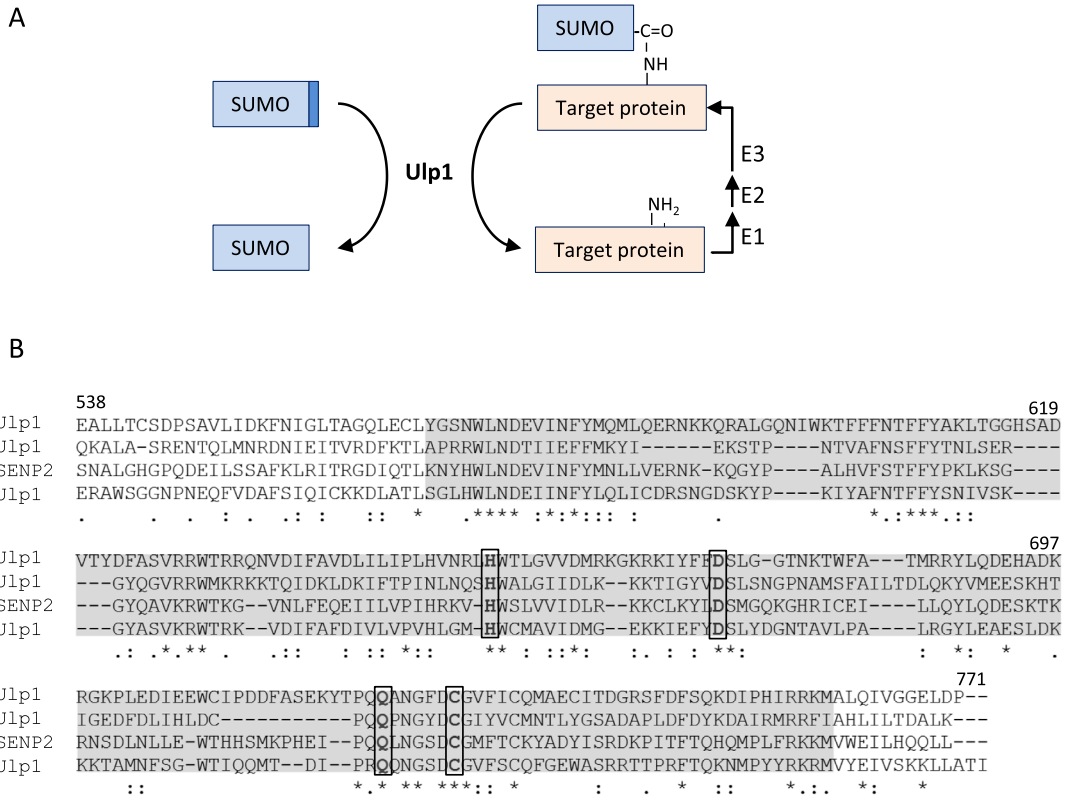

Fig. 1. Ubiquitin-like protease 1 (Ulp1) activity in the sumoylation and desumoylation pathways. (A) Ulp1 is a proteolytic enzyme responsible for two critical reactions of the sumoylation pathway. First, its proteolytic activity is responsible for the removal of amino acids from the carboxyl-terminus of an inactive precursor of small ubiquitin-like modifiers (SUMO) precursor to create a mature SUMO peptide (left reaction). Second, Ulp1 activity is required for the removal of a conjugated SUMO peptide from sumoylated proteins (right reaction). Following these reactions, the mature SUMO peptide can be used in the sumoylation of other target proteins. (B) Multiple amino acid sequence alignment of the C-terminal domain of Ulp1 homologs. The *Toxoplasma gondii* Ulp1 (*Tg*Ulp1, GenBank accession number EPR60181.1) amino acid sequence was aligned with Ulp1 homologs from yeast (*Saccharomyces cerevisiae*, *Sc*Ulp1, GenBank Accession number KZV07496.1), human (*Homo sapiens*, *Hs*SENP2, NP_067640.2) and *Caenorhabditis elegans* (*Ce*Ulp1, NP_498095.3). "*" represents conserved amino acid residues, ":" identifies amino acid residues with strongly similar properties, and "." is for weakly conserved amino acid residues. The grey highlighted area indicates the catalytic domain of Ulp1 homologs, whose essential residues for proteolytic activity are indicated by rectangles.

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