**Trypanosoma brucei** RAP1 Has Essential Functional Domains That Are Required for Different Protein Interactions

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**ABSTRACT** RAP1 is a telomere protein that is well conserved from protozoans to mammals. It plays important roles in chromosome end protection, telomere length control, and gene expression/silencing at both telomeric and nontelomeric loci. Interaction with different partners is an important mechanism by which RAP1 executes its different functions in yeast. The RAP1 ortholog in *Trypanosoma brucei* is essential for variant surface glycoprotein (VSG) monoallelic expression, an important aspect of antigenic variation, where *T. brucei* regularly switches its major surface antigen, VSG, to evade the host immune response. Like other RAP1 orthologs, *T. brucei* RAP1 (*TbRAP1*) has conserved functional domains, including BRCA1 C terminus (BRCT), Myb, MybLike, and RAP1 C terminus (RCT). To study functions of various *TbRAP1* domains, we established a strain in which one endogenous allele of *TbRAP1* is flanked by loxP repeats, enabling its conditional deletion by Cre-mediated recombination. We replaced the other *TbRAP1* allele with various mutant alleles lacking individual functional domains and examined their nuclear localization and protein interaction abilities. The N terminus, BRCT, and RCT of *TbRAP1* are required for normal protein levels, while the Myb and MybLike domains are essential for normal cell growth. Additionally, the Myb domain of *TbRAP1* is required for its interaction with *T. brucei* TTAGGG repeat-binding factor (*TbTRF*), while the BRCT domain is required for its self-interaction. Furthermore, the *TbRAP1* MybLike domain contains a bipartite nuclear localization signal that is required for its interaction with importin α and its nuclear localization. Interestingly, RAP1’s self-interaction and the interaction between RAP1 and TRF are conserved from kinetoplastids to mammals. However, details of the interaction interfaces have changed throughout evolution.

**IMPORTANCE** *Trypanosoma brucei* causes human African trypanosomiasis and regularly switches its major surface antigen, VSG, to evade the host immune response. VSGs are expressed from subtelomeres in a monoallelic fashion. *TbRAP1*, a telomere protein, is essential for cell viability and VSG monoallelic expression and suppresses VSG switching. Although *TbRAP1* has conserved functional domains in common with its orthologs from yeasts to mammals, the domain functions are unknown. RAP1 orthologs have pleiotropic functions, and interaction with different partners is an important means by which RAP1 executes its different roles. We have established a Cre-loxP-mediated conditional knockout system for *TbRAP1* and examined the roles of various functional domains in protein expression, nuclear localization, and protein-protein interactions. This system enables further studies of *TbRAP1* point mutation phenotypes. We have also determined functional domains of *TbRAP1* that are required for several different protein interactions, shedding light on the underlying mechanisms of *TbRAP1*-mediated VSG silencing.

**KEYWORDS** RAP1, telomere, *Trypanosoma brucei*, protein-protein interaction, RAP1
Telomeres are nucleoprotein complexes at linear chromosome ends. They are essential for genome integrity and chromosome stability (1). Telomere DNA in most eukaryotic cells consists of TG-rich tandem repeats (2), and proteins that associate with the telomere chromatin play critical roles in all aspects of telomere biology, including telomere length regulation (3) and protection of the natural chromosome ends from nucleolytic degradation and illegitimate DNA damage repair processes (1).

Among the core telomere protein components, RAP1 orthologs have been identified in many eukaryotes, including vertebrates (4–7), yeasts (8–15), and kinetoplastids (16). RAP1 orthologs have similar functions in chromosome end protection (17–27), telomere length control (4, 11, 17, 28–37), and telomeric silencing (12, 16, 34, 38–44) — where the expression of genes located at subtelomeric regions are suppressed by the heterochromatic telomere structure (45). Strikingly, RAP1 orthologs also have nontelomeric functions, including both transcription activation and repression activities (8, 46–55). In budding yeast, Saccharomyces cerevisiae RAP1 (ScRAP1) achieves different goals through interactions with various protein partners (50). For example, the C-terminal domain of ScRAP1 interacts with Sir3 and Sir4, which helps maintain telomeric silencing (39, 56, 57), and the same region of ScRAP1 also interacts with Rif1 and Rif2 to regulate the telomere length (58, 59). Interestingly, RAP1 orthologs have several conserved protein-protein interaction domains. All known RAP1s also have a BRCA1 C terminus (BRC) domain (4, 11, 12, 16) that is found in many proteins involved in DNA damage repair and replication (60, 61) and that frequently interacts with phosphorylated peptides (62–64). Human and yeast RAP1 orthologs also have a C-terminal conserved domain termed RAP1 C terminus (RCT) (4, 65), which is mainly involved in protein-protein interactions (39, 56–59, 66–68). All known RAP1 domains have a central Myb domain (4, 11–13, 16, 69). Although ScRAP1 uses its Myb and MybLike domains to bind duplex DNA directly (69), the human RAP1 Myb domain does not have any DNA binding activity, as its third helix has a negatively charged surface that is not suitable for DNA recognition (70).

A RAP1 ortholog has been identified in Trypanosoma brucei (16), a protozoan parasite that causes human African trypanosomiasis. T. brucei proliferates in extracellular spaces of its mammalian host and is directly exposed to the host immune surveillance. However, the parasite regularly switches its major surface antigen, variant surface glycoprotein (VSG), thereby effectively evading the host immune response (71). The T. brucei genome has >2,500 VSG genes and pseudogenes (72), which are all located at subtelomeres (72–74). VSGs are expressed exclusively from VSG expression sites (ESs), which are subtelomeric polycistronic transcription units transcribed by RNA polymerase I (RNA Pol I) (75, 76). VSG is the last gene in any ES, located within 2 kb of the telomere repeats, while the ES promoter is 40 to 60 kb upstream (73). There are 13 different ESs in the Lister 427 strain (74), all with the same gene organization and with ~90% sequence identity (73). However, at any given moment, only one ES is fully transcribed, presenting a single type of VSG on the cell surface (77). Monoallelic VSG expression ensures the effectiveness of VSG switching by avoiding presentation of a previously active VSG on the cell surface after a VSG switch, which helps the parasite to establish long-term infections. Many factors have been shown to regulate monoallelic VSG expression, including chromatin structure, transcription elongation, inositol phosphate pathway, and nuclear lamina (78, 79); a subtelomere and VSG-associated VEX complex (80, 81); and telomeric silencing (16, 44). VSG switching has two major pathways (82, 83). In an in situ switch, the originally active ES is silenced while a different one becomes fully active (82, 83). In recombination-mediated switches, either a silent VSG gene exchanges places with the originally active VSG without any loss of genetic information or a silent VSG gene is duplicated into the active ES to replace the originally active VSG gene (84). Many factors important for homologous recombination, DNA damage repair, and DNA replication influence VSG switching frequencies (84). Several telomere proteins also suppress VSG switching (85–88).

T. brucei RAP1 (TbRAP1) was identified as a factor interacting with T. brucei TTAGGG repeat-binding factor (TbTRF) (16), which binds the duplex telomere DNA directly (89).
Defining Protein Interaction Domains of TbRAP1

TbRAP1 is essential for cell proliferation, and depletion of TbRAP1 leads to a dramatic derepression of all ES-linked VSG genes up to >1,000-fold (16, 44). Transient depletion of TbRAP1 also results in an increased VSG switching frequency (88). Additionally, depletion of TbRAP1 leads to an increased level of the telomeric transcript (TERRA), an increased amount of telomeric RNA:DNA hybrids, and an elevated amount of telomeric/subtelomeric DNA damage (88). We showed that TbRAP1 has a BRCT domain located toward its N terminus, a central Myb domain, and a weak MybLike domain toward its C terminus (16). Here, we report that TbRAP1 also has a C-terminal RCT domain. However, functions of TbRAP1 domains are poorly understood. The interaction interface between TbRAP1 and TbTRF is unknown. Whether this interaction is required for the nuclear localization of TbRAP1 is unclear. These limitations have hindered further investigation of how TbRAP1 regulates VSG silencing and switching.

In this study, we established several strains in which one endogenous TbRAP1 allele is flanked by two loxP repeats so that it can be conditionally deleted by inducing Cre expression. Using this system, we determined that TbRAP1 Myb is necessary for TbTRF interaction and VSG silencing. Using TbRAP1 MybLike as bait in a yeast 2-hybrid screen, we have determined that importin α interacts with TbRAP1’s nuclear localization signal (NLS) residing in the MybLike domain. We found that TbRAP1 interacts with itself through the BRCT domain. In addition, the N terminus, BRCT, and RCT of TbRAP1 are required for normal TbRAP1 protein levels, while Myb and MybLike are essential for normal cell growth. These results not only provide further evidence of conserved essential functions of RAP1 orthologs throughout evolution but also pave the way for a better understanding of the mechanisms explaining how TbRAP1 silences subtelomeric VSG genes and helps maintain telomere stability and integrity.

RESULTS

Establishing a T. brucei strain with a floxed TbRAP1 allele. TbRAP1 is an essential protein (16), making it difficult to study phenotypes of various small deletions or point mutations using RNA interference (RNAi) (90). To better characterize functions of TbRAP1, we established a strain in which one TbRAP1 allele was flanked by two loxP repeats so that it was able to be conditionally deleted through Cre-mediated recombination (Fig. 1A). The other TbRAP1 allele can be replaced by various TbRAP1 mutants. Upon induction of Cre by the use of doxycycline, we are able to examine the phenotypes of the mutant TbRAP1, even if they are lethal.

A loxP site with the hygromycin resistance gene (HYG) and a loxP site with the blasticidin resistance gene (BSD) were targeted to locations upstream and downstream, respectively, of a given TbRAP1 allele (Fig. 1A). Both selectable markers were fused with the thymidine kinase gene (TK) from the herpes simplex virus, allowing selection for cells that had lost the floxed TbRAP1 allele (denoted as “F”) by the use of ganciclovir (GCV), as expression of the TK gene in T. brucei renders the parasite sensitive to GCV (91). To enable selection of cells that had lost the floxed allele by the use of fluorescence-activated cell sorting (FACS) analysis, we also fused the green fluorescent protein gene (GFP) with both selectable markers (Fig. 1A). Therefore, cells carrying a floxed TbRAP1 allele expressed HYG-GFP-TK and BSD-GFP-TK fusion proteins, and this was confirmed by Western analysis using a rabbit anti-GFP antibody (Life Technologies) in two TbRAP11/1 clones (without the Cre expression construct Cre-EP1 [91]) (see Fig. S1A in the supplemental material). Southern blotting also confirmed the genotype of these clones (Fig. S1B).

To validate that the floxed TbRAP1 allele can be excised by Cre efficiently, we transiently transfected the Cre-EP1 plasmid into TbRAP11/1 (-Cre-EP1) cells. Pools of cells and individual clones were selected with GCV. Western analysis performed with the GFP antibody showed that HYG-GFP-TK and BSD-GFP-TK were no longer expressed in either the pool or the clones (Fig. S1A). Southern analysis further confirmed that the floxed TbRAP1 allele was lost in the selected pool and clones (Fig. S1B).

To further increase the feasibility of conditional deletion of the floxed TbRAP1 allele, we integrated Cre-EP1 into a ribosomal DNA (rDNA) spacer region, whose expression
can be induced by doxycycline (91). The Cre-EP1-integrated \(TbRAP1^{F/+}\) cells grew normally in the presence of phleomycin, hygromycin, and blasticidin (Fig. S1C). Upon addition of doxycycline, these cells still grew normally in the presence of phleomycin only but did not survive in the presence of all three antibiotics (phleomycin, hygromycin, and blasticidin) (Fig. S1C), confirming that the doxycycline-induced Cre had excised the floxed \(TbRAP1\) allele together with the HYG-GFP-TK and BSD-GFP-TK markers. In the subsequent studies, the Cre-EP1 integrated \(TbRAP1^{F/+}\) strain was used.

**Conditional deletion of \(TbRAP1\) leads to cell growth arrest and VSG derepression.** In \(TbRAP1^{F/+}\) cells, we replaced the unfloxed \(TbRAP1\) allele with a pyrGomycin resistance marker (PUR) (Fig. 1A). In the resulting \(TbRAP1^{F/-}\) cells, addition of doxycycline led to the loss of the \(TbRAP1\) protein in 48 h (Fig. 1B), confirming the efficient deletion of the floxed \(TbRAP1\) allele. The \(TbRAP1\) mRNA level was estimated by quantitative reverse transcription PCR (RT-PCR), and two sets of \(TbRAP1\) primers were used: one annealed specifically to the N-terminal region and another annealed to the BRCT domain (Fig. 1A). After 24 h of Cre induction, the \(TbRAP1\) mRNA level dropped to 10% of the wild-type (WT) level in \(TbRAP1^{F/-}\) cells (Fig. 1C), indicating that the sole \(TbRAP1\) allele in these cells was excised by Cre. In contrast, the \(TbRAP1\) mRNA level dropped to \(-50%\) of the WT level in \(TbRAP1^{F/+}\) cells (Fig. 1C), as only the floxed \(TbRAP1\) allele had been deleted, leaving the other WT allele intact.

We previously showed that depletion of \(TbRAP1\) by RNAi led to cell growth arrest and VSG derepression (16, 44). Induction of Cre in \(TbRAP1^{F/-}\) cells also led to a severe growth defect (Fig. 1D). Quantitative RT-PCR analysis showed that ES-linked VSG6, VSG8, VSG9, VSG11, VSG591, and VSG639 (all originally silent) were derepressed by several 10-fold orders after induction of Cre for 24 h and by several 100-fold orders after 48 h (Fig. 1E). As a control, the rRNA levels and mRNA levels of two chromosome-internal genes, SNAP50 and \(Tb11.0330\), did not increase significantly (Fig. 1E). The mRNA level of the originally active VSG2 gene was decreased \(-20%\) (Fig. 1E). Therefore, conditional deletion of \(TbRAP1\) by Cre-loxP exhibited the same phenotypes as depletion of \(TbRAP1\) by RNAi (16), indicating that the Cre-loxP-mediated conditional deletion is feasible and efficient.
The TbRAP1 Myb domain is essential for normal cell growth and VSG silencing.

We previously showed that TbRAP1 has a BRCT domain, a Myb domain, and a MybLike domain (Fig. 2, top) (16). With a careful sequence analysis, we found that the C terminus of TbRAP1 has recognizable similarities to the RCT domains of other RAP1 orthologs (Fig. 2, bottom). The level of sequence identity between TbRAP1 and other RAP1 orthologs in this domain was 11.7%, which is approximately the same as that in the BRCT domain (16). We named this region “RCT.” Therefore, TbRAP1 has several conserved domains like other known RAP1 orthologs (Fig. 2, top).

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other genes, we performed transcriptome sequencing (RNA-seq) analysis. Both $TbRAP1^{+/-}$ and $TbRAP1^{+/F2H-∆Myb}$ cells were induced for Cre expression for 30 h, after which the total RNA was isolated. Poly(A) RNA was purified and used for library construction at Novogene followed by paired-end high-throughput sequencing using Illumina (Materials and Methods). Differential gene expression analysis showed that more than 8,000 genes were upregulated and nearly 3,000 genes were downregulated in Cre-induced $TbRAP1^{+/F2H-∆Myb}$ cells compared to the results seen with $TbRAP1^{+/-}$ (Fig. 3F). However, the fold change in mRNA levels was much greater for upregulated
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F2H-TbRAP1ΔMybL:VSG2:DNA

F2H-NLS-TbRAP1ΔMybL:VSG2:DNA

FIG 4 Immunofluorescent analysis of TbRAP1^WT/TbRAP1ΔMybL TRFi and TbRAP1^WT/TbRAP1ΔMybL TRFi cells. F2H-TbRAP1ΔMybL and F2H-NLS-TbRAP1ΔMybL were stained with 12CA5 HA antibody. A rabbit VSG2 antibody was used to show the outline of the cell body. DNA was stained with DAPI. In each strain, three different cells are shown in three panels.

genes than for downregulated ones (Fig. 3F), and upregulated genes were present in much greater numbers than downregulated ones, suggesting that TbRAP1 has a major role in gene silencing and a minor role in gene activation. Sequence read coverage in all VSG bloodstream-form (BF) expression sites (BESs) (74) showed that all silent BES-linked VSG genes and some BES-linked expression site-associated genes (ESAG genes) were upregulated, but other BES-linked ESAG genes were not affected or were even downregulated (Fig. S3). Based on available annotation of the affected genes, a total of more than 2,700 VSG genes and pseudogenes were upregulated (Fig. S2C), which included nearly all reported VSG genes/pseudogenes in the Lister 427 genome (72). Therefore, the TbRAP1 Myb domain is essential for the functions of TbRAP1 in normal cell growth and VSG silencing. Interestingly, the mRNA levels of some ribosomal protein genes were decreased in the mutant, although at only up to 60% of the normal level (Fig. S2D). It is possible that TbRAP1 may also participate in transcription activation of ribosomal protein genes, as was seen previously with ScRAP1 (8), although further investigation is necessary to validate this.

The nuclear localization signal of TbRAP1 is required for its interaction with Importin α and nuclear localization. Using the same approach, we replaced the WT TbRAP1 allele with an F2H-tagged TbRAP1 mutant lacking the MybLike domain (TbRAP1ΔMybL). Southern blotting confirmed its genotype (Fig. S4A). Western blotting showed that F2H-TbRAP1ΔMybL was expressed (Fig. S4B) and that the expression was at the same level as F2H-TbRAP1 (Fig. 3B). However, IF showed that this mutant was localized in the cytoplasm (Fig. 4A). Using Motif Scan analysis (https://myhits.isb-sib.ch/cgi-bin/motif_scan) (92), we found that the sequence consisting of amino acids (aa) 727 to 741 of TbRAP1 represents a bipartite nuclear localization signal (NLS). F2H-TbRAP1ΔMybL lacks this NLS, which is likely why this mutant is localized in the cytoplasm. To confirm this, we added the simian virus 40 (SV40) large T NLS to the N terminus of TbRAP1ΔMybL (Fig. 3A). Southern and Western analyses confirmed the genotype of this strain and that the expression of F2H-NLS-TbRAP1ΔMybL was at the WT level (Fig. 3B; see also Fig. S4C and D). IF analysis showed that F2H-NLS-TbRAP1ΔMybL was indeed localized in the nucleus (Fig. 4B).
To further explore how TbRAP1 is imported into the nucleus, we screened a normalized yeast 2-hybrid library generated from T. brucei cDNA using the TbRAP1 MybLike domain as bait. In this screen, 16.5 million yeast primary transformants were obtained, and a total of 711 clones were positive in the initial screen. The majority of the candidates represented the same gene, Tb427.06.2640, which is annotated as encoding the importin/H9251 subunit in TriTrypDB (93, 94). The canonical function of importin/H9251 is to bind the NLS of nuclear proteins, form a complex with importin/H9252, and transport the protein into the nucleus through the nuclear pore (95). Once inside the nucleus, importin/H9251 releases its cargo and exits the nucleus to transport the next cargo (95).

TbRAP1 MybLike containsthe predicted bipartite NLS. Therefore, we expected that importin/H9251 would interact with the TbRAP1 NLS and this interaction would be essential for transporting TbRAP1 into the nucleus.

To confirm the interaction between importin α and TbRAP1, we inserted a C-terminal myc13 (13 repeats of myc) epitope at one endogenous importin α allele. PCR analysis confirmed correct targeting in three different TbRAP1 backgrounds: both alleles were WT, one of the two WT alleles had an N-terminal F2H tag, and one of the alleles was replaced with the TbRAP1F2H-ΔMybL mutant (Fig. S5). The latter two strains also carried an inducible TbTRF RNAi cassette inserted into an rDNA spacer, although the RNAi was not induced for the analysis of TbRAP1-importin α interaction. The expression of importin α-myc13 was confirmed by Western blotting (Fig. 5A). Subsequently, we performed coimmunoprecipitation (co-IP) experiments in these three strains. In the

![Image](msphere.asm.org/8)

**FIG 5** The TbRAP1 MybLike domain is required for interaction with importin α. (A) Expression of importin α-myc13 in TbRAP1+/+, TbRAP1F2H+/+, TRFi, and TbRAP1F2H-ΔMybL+/+ TRFi cells. Myc13-tagged proteins were detected by a myc monoclonal antibody, 9E10 (MSKCC monoclonal antibody core). (B) Co-IP of TbRAP1 and importin α. The myc antibody 9E10, an anti-TbRAP1 rabbit antibody (16), and IgG (as a negative control) were used for IP in TbRAP1+/+ cells. Western analysis was performed using the antibodies mentioned above to detect importin α-myc13 and TbRAP1. In TbRAP1F2H+/+, TRFi and TbRAP1F2H-ΔMybL+/+ TRFi cells, the 9E10 myc antibody, the 12CA5 HA antibody, and IgG (as a negative control) were used for IP, and Western blotting was performed to detect importin α-myc13 (by 9E10) and F2H-tagged WT and mutant TbRAP1 (by 12CA5 in the left panels and HA probe in the right panels). In this and other figures, input samples represent 1% of the materials used for IP.
**TbRAP1**^+/+ background, IP experiments were performed using a rabbit *TbRAP1* antibody (16) or the myc monoclonal antibody 9E10 (Memorial Sloan Kettering Cancer Center [MSKCC] monoclonal antibody core). In *TbRAP1^F2H/+* TRF RNAi (TRFi) and *TbRAP1^F2H-ΔMybL/+* TRFi cells, IP experiments were performed using HA monoclonal antibody 12CA5 (MSKCC monoclonal antibody core) or 9E10. In cells carrying untagged or F2H-tagged WT *TbRAP1*, both importin α-myc, and *TbRAP1* were present in the IP products (Fig. 5B, top two rows). However, F2H-*TbRAP1ΔMybL* and importin α-myc were not in the same IP product (Fig. 5B, bottom row). Therefore, the MybLike domain of *TbRAP1* (and most likely the NLS in this domain) is necessary for *TbRAP1*’s interaction with importin α and its nuclear localization.

**The *TbRAP1* Myb domain interacts with *TbTRF*.** *TbRAP1* was originally identified as a *TbTRF*-interacting factor in a yeast 2-hybrid screen (16). The N-terminal part of *TbRAP1* (including the N terminus, BRCT, and Myb) is sufficient to interact with *TbTRF* in yeast 2-hybrid analysis (16), and WT *TbRAP1* and *TbTRF* co-IP in vivo (16), although both assays showed a weak interaction between the two proteins. To examine which *TbRAP1* functional domain(s) is essential for the *TbRAP1-* *TbTRF* interaction, in the *TbRAP1^F/+/F2H^+* cells, we either targeted an F2H tag to the N terminus of the WT *TbRAP1* allele (to generate the *TbRAP1^F/F2H^+* strain) or replaced the WT allele with an F2H-tagged *TbRAP1* mutant lacking various functional domains (to generate the *TbRAP1^F/F2H-mut^* strains) (Fig. 3A). Southern analyses confirmed the replacement of the WT *TbRAP1* allele by the *TbRAP1^F2H-ΔNT^*, *TbRAP1^F2H-ΔBRCT^*, or *TbRAP1^ΔMybΔRCT-F2H-NLS^* allele in the corresponding strains (Fig. 5A to C). Here, we added the second half (aa 736 to 742) of the endogenous *TbRAP1* NLS (labeled as NLSr) at the C terminus of *TbRAP1ΔMybΔRCT*. These *TbRAP1* mutants were expressed (Fig. 6A) but at much lower levels than F2H-*TbRAP1* (Fig. 3B). F2H-*TbRAP1ΔNT* and F2H-*TbRAP1ΔBRCT* still contained the *TbRAP1* NLS, and they were localized in the nucleus as expected (Fig. 6B, left and middle). Interestingly, *TbRAP1ΔMybΔRCT-F2H-NLS* was also localized in the nucleus (Fig. 6B, right), indicating that the sequence consisting of aa 736 to 742 contains a minimum nuclear localization signal and that its presence is sufficient to target *TbRAP1* to the nucleus. This observation further validated the function of *TbRAP1* NLS.

Since human RAP1 interacts with itself (4), it is possible that *TbRAP1* may also interact with itself (see below). To avoid detecting possible indirect interactions between *TbTRF* and mutant *TbRAP1* mediated by the WT *TbRAP1*, we induced Cre in these *TbRAP1^F/F2H-mut^* strains for 30 h to ensure the depletion of the WT *TbRAP1* protein (Fig. 6A). Depletion of *TbRAP1* by RNAi for up to 36 h still allows complete cell growth recovery 24 h after removal of the RNAi induction (88). In addition, upon Cre induction, the number of *TbRAP1^F/^* and *TbRAP1^F/F2H-mut^* cells did not decrease for several days (Fig. 1D) (Fig. 3D; see also Fig. 5D to G). Therefore, inducing *TbRAP1^F/F2H-mut^* for 30 h caused only cell growth arrest rather than cell death. Subsequently, co-IPs were performed using a rabbit *TbTRF* antibody (89). In all cases, *TbTRF* was detected in the IP products by Western analysis using a chicken *TbTRF* antibody (16) (Fig. 6C, right). Although F2H-*TbRAP1ΔNT*, F2H-*TbRAP1ΔBRCT*, and *TbRAP1ΔMybΔRCT-F2H-NLS* were expressed at very low levels (Fig. 3B), these mutants still interacted with *TbTRF*, as they were detected in the IP products in the same manner as F2H-*TbRAP1* and F2H-NLS-*TbRAP1ΔMybL* (Fig. 6C, left). However, F2H-*TbRAP1ΔMybL* was not detected in the IP product (Fig. 6C, left). In addition, IF analysis showed that F2H-*TbRAP1ΔMyb* was not colocalized with *TbTRF* even though these proteins were in the nucleus (Fig. 6D). As a control, WT *TbRAP1* was partially colocalized with *TbTRF* (Fig. 6D), as we have shown previously (16). Therefore, the Myb domain of *TbRAP1* is required for its interaction with *TbTRF*. Interestingly, *TbRAP1^F/F2H-NLS-ΔMybL^* cells exhibited a growth arrest phenotype after induction of Cre for 30 h (Fig. 5A), even though F2H-NLS-*TbRAP1ΔMybL* was expressed at the WT level (Fig. 3B) and was localized in the nucleus (Fig. 4B), indicating that the MybLike domain has essential functions other than transporting *TbRAP1* into the nucleus. On the other hand, F2H-*TbRAP1ΔNT*, F2H-*TbRAP1ΔBRCT*, and
Fig. 7B

F2H-TbRAP1 antibody (antibody, and the IP products were examined by Western blotting using both the HA TbRAP1 probe antibody and the WT TbRAP1 antibody [16]) before and after induction of Cre (Fig. S6E to G). The antibodies used for Western analyses of the IP products were the 12CA5 HA antibody (except for TbRAP1ΔNT, where the HA probe was used) and an anti-TRF chicken antibody (16). (D) (Top) F2H-TbRAP1 mutants (using the HA probe antibody) and WT TbRAP1 mutants (as labeled on the left). (Bottom) F2H-TbRAP1ΔMybLΔRCT-F2H-NLS cells (detected by a rabbit TRF antibody) and F2H-TbRAP1ΔMybLΔRCT-F2H-NLS cells (detected by a rabbit TRF antibody [16]). DNA was stained with DAPI. Three different TbRAP1ΔNT/ΔMybLΔRCT-F2H-NLS cells are shown.

TbRAP1ΔMybLΔRCT-F2H-NLS, were expressed at much lower levels than WT TbRAP1 (Fig. 3B), indicating that the N terminus and BRCT and RCT domain of TbRAP1 are required for normal TbRAP1 protein levels, which is likely the reason why TbRAP1ΔMybLΔRCT-F2H-NLS cells also showed a growth arrest phenotype after induction of Cre (Fig. S6E to G).

The BRCT domain is required for TbRAP1 self-interaction. To test whether TbRAP1 has any self-interaction ability, we performed co-IP experiments in cells expressing an F2H-TbRAP1 from one of its endogenous alleles. Since TbRAP1 interacts with TbTRF, these co-IP experiments were performed in TbTRF RNAi cells. Both before and after depletion of TbTRF by RNAi (Fig. 7A, right), we detected WT TbRAP1 in the IP product when IP was performed using the 12CA5 HA antibody (Fig. 7A, left). Therefore, TbRAP1 interacts with itself, and this interaction is independent of TbTRF.

To further examine which domain of TbRAP1 is required for its self-interaction, we tested whether any F2H-tagged TbRAP1 domain deletion mutants showed co-IP with the WT TbRAP1. In TbRAP1ΔNT cells (without Cre induction) and in TbRAP1ΔMybLΔRCT-F2H-NLS cells, IP experiments were performed using the 12CA5 HA antibody, and the IP products were examined by Western blotting using both the HA TbRAP1 probe antibody (Fig. 7B, right) and the rabbit TbRAP1 antibody (16) (Fig. 7B, left). In all cells except TbRAP1ΔNT, WT TbRAP1 was detected in the IP products (Fig. 7B, left), indicating that BRCT is essential for TbRAP1 self-interaction. Although F2H-
Defining Protein Interaction Domains of *TbRAP1*

**FIG 7** *TbRAP1* interacts with itself through the BRCT domain. (A) *TbRAP1*’s self-interaction activity is independent of *TbTRF*. Left, co-IP experiments were done using the 12CA5 HA antibody and IgG (as a negative control) in *TbRAP1*Δ2H+/− TRFi cells before and after induction of *TbTRF* RNAi. IP products were analyzed by Western blotting using a rabbit *TbRAP1* antibody (16). To differentiate F2H-*TbRAP1* and the untagged *TbRAP1*, proteins were separated on a 7.5% Tris polyacrylamide gel for 7 h 40 min. Right, Western blotting using an anti-*TbRAP1* rabbit antibody (16), and other F2H-tagged *TbRAP1* mutants were detected by the HA probe antibody in Western blotting. To differentiate *TbRAP1* and F2H-*TbRAP1*ΔMyb, proteins were separated on a 7.5% Tris polyacrylamide gel for 7 h 40 min.

*TbRAP1*ΔBRCT was expressed at a lower-than-WT level, it was expressed at a higher level than F2H-*TbRAP1*ΔNT and *TbRAP1*ΔMybΔRCT-F2H-NLS, (Fig. 3B), while the latter two mutants interacted with the WT protein (Fig. 7B, left). Therefore, the lack of interaction between F2H-*TbRAP1*ΔBRCT and WT *TbRAP1* is unlikely to have been due to the low level of expression of the mutant.

**DISCUSSION**

RAP1 orthologs are conserved from protozoa to mammals (4–16), and they have similar domain structures (4, 12, 16, 65) and essential telomeric and nontelomeric functions (96). *TbRAP1* also has the BRCT, Myb, MybLike, and RCT functional domains, like other RAP1 orthologs (16). *TbRAP1* is essential for VSG silencing and telomere/subtelomere integrity and stability (16, 44, 88). However, whether *TbRAP1*’s domains are required for these functions was unknown. Study of *TbRAP1* domain functions was partly limited by the fact that *TbRAP1* is essential for cell proliferation (16). Previous studies of *TbRAP1* functions were heavily dependent on the use of conditional RNAi to deplete *TbRAP1* (16, 44, 88). Although expressing double-stranded RNA (dsRNA) of the *TbRAP1* full-length gene (16, 88), the BRCT fragment, or the RCT fragment (44) can efficiently deplete *TbRAP1*, expressing dsRNA of the 3’ untranslated region (3’UTR) of *TbRAP1* cannot. Therefore, the RNAi approach is not suitable for studying phenotypes of all domain deletion mutants or point mutations of *TbRAP1*. In this study, we took advantage of the Cre-loxP system (91) and established a series of strains in which one endogenous *TbRAP1* allele is flanked by two repeats of loxP, allowing its conditional deletion upon Cre induction. We confirmed that this conditional deletion was able to efficiently deplete the *TbRAP1* protein and mRNA. Most importantly, we are now able to examine the phenotypes of a series of *TbRAP1* mutants that lack individual functional domains or carry point mutations, even if the mutants are lethal.
In this study, we found that none of the domain deletion mutants of TbrAP1 could support normal cell growth. F2H-TbrAP1ΔNT, F2H-TbrAP1ΔBRCT, and TbrAP1ΔMybΔRCT-F2H-NLS were expressed at much lower levels than the WT TbrAP1, while F2H-TbrAP1ΔMyb, F2H-TbrAP1ΔMybL, and F2H-NLS-TbrAP1ΔMybL were expressed at the same level as WT TbrAP1. Therefore, the N terminus, BRCT, and RCT are required for normal TbrAP1 protein level, and the low level of protein expression is most likely the reason why these mutants do not support normal cell growth. F2H-TbrAP1ΔMyb and F2H-NLS-TbrAP1ΔMybL were localized in the nucleus and expressed at the same level as the WT protein, but these mutants still had a severe growth defect, indicating that Myb and MybLike domains are essential for normal cell growth. Only the DNA binding domains of ScRAP1 (Myb and MybLike) are essential for cell viability, and its RCT domain, which is important for telomere length regulation and telomeric silencing, is not essential for cell survival (66). This leads us to speculate that the TbrAP1 Myb and/or MybLike domains may have DNA binding activities. Although Myb domains are frequently involved in DNA binding (97), the human RAP1 Myb domain does not seem to have any DNA binding activity due to its negative surface charge on the third helix, which is typically involved in DNA recognition (70). In addition, the ScRAP1 MybLike domain was revealed to fold into a DNA binding motif only after its crystal structure was solved (69). It will be interesting to investigate whether TbrAP1 Myb and MybLike domains have any DNA binding activities, but further protein structural analysis may be necessary.

Myb domains can also mediate protein-protein interactions (98, 99). We found that TbrAP1 Myb interacts with TbrTRF, providing another piece of evidence that the Myb domain can have an important function in protein-protein interaction. Nevertheless, whether the interaction between TbrAP1 and TbrTRF is essential for cell survival and/or VSG silencing is still unknown. Further investigation will be necessary to identify key residues in TbrAP1 Myb that are critical for TbrTRF interaction, which will help address this issue. Human RAP1 uses its C-terminal RCT domain to interact with TRF2’s linker region (4, 68). Similarly, Schizosaccharomyces pombe RAP1 also uses its C-terminal RCT domain to interact with Taz1, a functional homologue of mammalian TRF1/2 (65, 68), indicating that this interaction interface is conserved from yeast to mammals. However, we found that the TbrAP1 Myb domain is critical for TbrTRF interaction. It is interesting that the RAP1-TRF interaction is preserved from kinetoplastids to mammals and yet the functional domains that accomplish this goal have changed.

The transcription profile of more than 10,000 genes was found to have changed in TbrAP1 ΔMyb cells after induction of Cre. In particular, ~2,700 VSG genes were upregulated. Since the VSGnome identified more than 2,500 VSG genes and pseudo-genes in the Lister 427 genome (72), this means nearly all of the VSG genes were upregulated, further validating that TbrAP1 has an essential function in silencing VSG genes and that Myb is essential for this function. Interestingly, RNA-seq also identified nearly 3,000 genes that are downregulated in TbrAP1 ΔMyb cells, although the fold change in mRNA levels was much lower than that seen with the upregulated genes. ScRAP1 has been well known for both its transcription activation and its repression functions (50), and ScRAP1 is required for ribosomal protein gene activation (8). Our observation suggests that TbrAP1 may have functions similar to those of ScRAP1, although TbrAP1’s transcription activation effect appears to be much weaker than its repressive effect. Some of the downregulated genes encode ribosomal proteins, suggesting that TbrAP1 may also participate in ribosomal protein gene activation. However, further validation is necessary to confirm TbrAP1’s role as a transcription activator.

Importing >45-kDa nuclear proteins frequently depends on the presence of NLS, which can be recognized by importin α/β proteins (100). After the importin-cargo complex is transported through the nuclear pore complexes, binding of RanGTP to the importin β dissociates the complex to release cargo into the nucleoplasm (100). However, nuclear proteins can also be transported into the nucleus through NLS-independent mechanisms, such as interacting with a protein partner that contains a NLS (101). TbrAP1 is a nuclear protein (16). However, how TbrAP1 is imported into the
nucleus was unknown. Here, we show that importin α directly interacts with TbRAP1 and that this interaction depends on the MybLike domain, which contains a predicted bipartite NLS. Additionally, F2H-TbRAP1ΔMybL is not localized in the nucleus, while TbRAP1ΔMybLΔRCT-F2H-NLS, with the second half of the TbRAP1 NLS, is. Therefore, TbRAP1’s nuclear localization depends on the NLS in the MybLike domain and requires its recognition by importin α. Addition of an SV40 large T NLS to the N terminus of TbRAP1ΔMybL can target the mutant to the nucleus, indicating that the NLS and importin α are well conserved with other known classical NLS and importin α proteins, respectively. Interestingly, F2H-NLS-TbRAP1ΔMybL still interacts with TbTRF. However, without any NLS, F2H-TbRAP1ΔMybL is localized in the cytoplasm, indicating that the interaction between TbRAP1 and TbTRF is not sufficient to bring TbRAP1 into the nucleus. Human RAP1 interacts with itself through the RCT domain (4), although the function of this self-interaction is unknown. Human RAP1 interacts tightly with TRF2 with equal stoichiometry (102), and TRF2 homodimerizes (103). It is possible that homodimerization of human RAP1 allows a better interaction with TRF2. The TbTRF-TbRAP1 interaction is much weaker than the TbTRF-TbTIF2 interaction (4, 86), and whether TbTRF interacts with TbRAP1 with equal stoichiometry is unknown. Therefore, whether TbRAP1 self-interaction contributes to its interaction with TbTRF is not clear. We found that TbRAP1’s self-interaction is independent of TbTRF, indicating that this self-interaction is direct and not mediated by the TbRAP1-TbTRF interaction. Additionally, TbRAP1 self-interaction and TbRAP1-TbTRF interaction require different functional domains. The BRCT domain is also important for a normal level of TbRAP1 protein. It is possible that TbRAP1 is better stabilized with self-interaction, possibly by preventing TbRAP1 degradation by proteases. Further analysis with point mutations in the BRCT domain that specifically abolish TbRAP1 self-interaction will be useful to reveal its function and help understand the mechanism of protein stabilization.

The fact that the functional interactions of RAP1 with other telomere proteins, such as TRF, or with itself are conserved from kinetoplastids to mammals suggests that these functions of RAP1 orthologs are critical for essential cellular processes. However, throughout evolution, different organisms have used different approaches to achieve the same goal. Hence, the detailed protein-protein interaction interfaces have been changed even though the consequential protein complex is still preserved. We have observed a similar scenario in interactions of TbTRF and TbTIF2 (86). Although TbTRF interacts with TbTIF2 (86) and mammalian TRF1 and TRF2 interact with TIN2 (104, 105), the two protein pairs interact with different interfaces (86). Therefore, conserved protein-protein interactions without a conserved interaction interface can be common among many telomere protein homologues.

TbRAP1, as with its orthologs in other organisms, has multiple protein-protein interaction domains. A common theme appears to pertain for most RAP1 orthologs: RAP1 interacts with different protein partners to perform different cellular tasks (50). Our results further suggest that TbRAP1 interacts with different partners through different functional domains to achieve various goals. With our established Cre-loxP system, we will be able to investigate details of the functions of each TbRAP1 domain in the future.

MATERIALS AND METHODS

T. brucei strains and plasmids. All T. brucei strains used in this study (listed in Table S1 in the supplemental material) were derived from bloodstream-form Lister 427 cells that express VSG2 as well as a T7 polymerase and the Tet repressor (also known as the single marker or SM strain) (106). All BF T. brucei cells were cultured in HMI-9 medium supplemented with 10% fetal bovine serum (FBS) and appropriate antibiotics.

To establish the TbRAP1 Δ+ Δ- strain, the NotI/XhoI-digested TbRAP1 5′-UTR-loxP-HYGGFPTK plasmid and NotI/XhoI-digested TbRAP1 3′-UTR-BSDGFPTK-loxP plasmid were sequentially transfected into SM cells. The A1 and A3 clones of TbRAP1 Δ+ Δ- (-Cre-EP1) were confirmed by Western and Southern analyses (see Fig. S1A and B in the supplemental material). The conditional Cre-expressing Cre-EP1 plasmid with the phleomycin resistance (BLE) marker (91) was subsequently inserted into an rDNA spacer region to
To establish the TbRAP1Δ/Δ strain, the NotI/Xhol-digested pSK-TbRAP1-ko-PUR plasmid was transfected into TbRAP1Δ/Δ cells. The pSK-TbRAP1-ko-PUR plasmid contains PUR flanked by sequences upstream and downstream of the TbRAP1 open reading frame (ORF), respectively.

All TbRAP1Δ/Δ-mut strains were established using the same strategy. N-terminal F2H-tagged TbRAP1ΔNT, TbRAP1ΔBRCT, TbRAP1ΔMybL, and NLS-TbRAP1ΔMybL mutants flanked by sequences upstream and downstream of the TbRAP1 gene, together with a PUR marker, were cloned into pSK to generate respective targeting constructs. The TbRAP1ΔMybLΔRCT-F2H-NLS, mutant flanked by sequences upstream and downstream of the TbRAP1 gene was also cloned into pSK with a PUR marker to generate the mutant targeting construct. All mutant targeting plasmids were digested with SacII (or with PvuII in the case of BMYbLΔRCT) before transfection of the TbRAP1Δ/Δ cells was performed to generate the corresponding TbRAP1Δ/Δ-mut strains. All mutant strains were confirmed by Southern analyses.

For examination of TbRAP1 self-interaction in the presence and absence of TbTRE, a TbTRE RNAi (TRFi) strain was first established by transfection of the NotI-digested pZJM-3-TbTRE-Mid1 RNAi construct (89) into SM cells. Subsequently, one endogenous TbRAP1 allele was tagged with an N-terminal F2H tag by transfection of a SacII-digested pSK-PUR-F2H-TbRAP1-tar2 construct into the TRFi cells.

Insertion of 13 C-terminal repeats of myc into one endogenous allele of importin α was performed in TbRAP1Δ/Δ, TbRAP1Δ/Δ-mut TRFi, and TbRAP1Δ/Δ-mut TRFi cells. TbRAP1Δ/Δ-mut TRFi cells were obtained by replacing one endogenous TbRAP1 allele with the F2H-TbRAP1ΔMybL mutant in TRFi cells as described above. (The TbRAP1Δ/Δ-mut TbRAP1Δ/Δ-mut TRFi strain used for TbRAP1 co-IP analysis was established in a similar way.) The importin α C-terminal myc13 tagging fragment was amplified by PCR with primers OBL-TbIMP10-C-CT-MYC-FW (5'-GAGGGGCACTCAGCAGTTTGAGTTGGGGATG-3') and OBL-TbIMP10-C-CT-MYC-BW (5'-GATATATATATACCAAGGTGCCTCCCTGCTACCATTTGATCTCGT-3') by the use of a plasmid containing myc13, and a HYG marker as the template. The PCR product was then purified and transfected into TbRAP1Δ/Δ, TbRAP1Δ/Δ-mut TRFi, and TbRAP1Δ/Δ-mut TRFi cells.

**Combinonucleoprecipitation.** A total of 200 million T. brucei cells in log-phase growth were used for each IP using appropriate antibody or IgG (as a negative control). IPs were pulled down by the use of Dynabeads protein G (Life Technologies) and split equally for two Western blotting analyses using appropriate antibodies. A 1% volume of input sample was loaded as a control. Since F2H-TbRAP1ΔNT, F2H-TbRAP1ΔBRCT, and TbRAP1ΔMybLΔRCT-F2H-NLS, express at low levels, 500 million mutant cells were used for each IP. A 1% volume of input was loaded in Western analysis as a control.

Immunofluorescence analyses.** If experiments were performed as described previously (86). Specifically, cells were fixed with 2% formaldehyde at room temperature for 10 min, permeabilized in 0.2% NP-40–1× phosphate-buffered saline (PBS) at room temperature for 5 min and blocked by the use of 1× PBS–0.2% cold fish gelatin–0.5% bovine serum albumin (BSA) at room temperature twice for 10 min each time, followed by incubation with the primary antibody at room temperature for 2 h and the secondary antibody at room temperature for 1 h. Cells were then washed with 1× PBS–0.2% cold fish gelatin–0.5% BSA and 1× PBS followed by staining with 0.5 μg/ml DAPI (4′,6-diamidino-2-phenylindole) and by mounting of coverslips on slides. Images were taken by a DeltaVision Elite deconvolution microscope. Images were deconvolved using SoftWoRx.

**T. brucei cDNA library.** A normalized T. brucei cDNA library was prepared by Bio S&T. Briefly, using a modified SMART cDNA synthesis method, the total RNA from WT T. brucei cells was used for synthesis of cDNA with either oligo(dT) or a random primer. The cDNA was normalized and amplified, after which it was inserted into a modified pGAD T7 yeast expression vector.

**Yeast 2-hybrid screen.** The TbRAP1 Myb-like domain was cloned into the pBTM116 vector and transformed into the yeast strain L41 [MATa his 3D200 trp1-901 leu2-3,112 ade2 lys::(LexAop)4-HIS3 URA3::(LexAop)8-loa2 gal1 gal10]. The resulting cells were transformed with the normalized T. brucei cDNA library. A total of 16.5 million primary transformants were plated onto synthetic drop-out (SD) plates without tryptophan, leucine, or histidine. A total of 711 clones were obtained from this initial screening.

**RNA-seq.** Cre expression was induced by the use of doxycycline in TbRAP1Δ/Δ-mut and TbRAP1Δ/Δ cells for 30 h, after which total RNA was isolated and purified through RNeasy columns (Qiagen). Three independent inductions were performed as biological replicates. RNA samples were run on a BioAnalyzer 2100 system (Agilent Technologies) using an Agilent RNA 6000 Nano kit to verify the RNA quality and then sent to Novogene for library preparation and RNA high-throughput sequencing.

The following processes were performed at Novogene.

(i) **RNA quantification and qualification.** RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using a NanoPhotometer spectrophotometer (Implen). RNA integrity and quantitation were assessed using an RNA Nano 6000 assay kit on a Bioanalyzer 2100 system (Agilent Technologies).

(ii) **Library preparation for transcriptome sequencing.** Sequencing libraries were generated using a NEBNext Ultra RNA library prep kit for Illumina (NEB, USA) using 1 μg poly(A) RNA according to the manufacturer’s protocol, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly(T) oligonucleotide-attached magnetic beads.

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tion was carried out using divalent cations under conditions of elevated temperature in NEBNext first-strand synthesis reaction buffer (5×). First-strand cDNA was synthesized using random hexamer primers and Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RNase H–). Second-strand cDNA synthesis was performed using DNA polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with a hairpin loop structure was ligated to prepare for hybridization. In order to select cDNA fragments preferentially of length 150 to 200 bp, the library fragments were purified with an AMPure XP system (Beckman Coulter). A 3-μl volume of USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C followed by PCR, which was performed with Phusion high-fidelity DNA polymerase, universal PCR primers, and an Index (X) primer. PCR products were purified (AMPure XP system), and library quality was assessed on an Agilent Bioanalyzer 2100 system.

(iii) Clustering and sequencing (Novogene Experimental Department). Clustering of the indexed-copies samples was performed on a cBot cluster generation system using a cBot-HiSeq (HS) paired-end (PE) cluster kit (Illumina) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and 125-bp and 150-bp paired-end reads were generated.

RNA-seq data analysis. The RNA-seq data were analyzed by Novogene as follows.

(i) Quality control. Raw reads of fastq format were first processed through the use of Novogene perl scripts. In this step, clean reads were obtained by removing reads containing adapters, reads containing poly(N), and low-quality reads. At the same time, the levels of Q20, Q30, and GC content of the clean reads were calculated. Subsequent downstream analyses were performed on the basis of the clean reads with high quality.

(ii) Mapping of reads to the reference genome. The T. brucei Lister 427 genome TriTrypDB-45_T.bruceiLister427_2018.Genome.fasta and its annotation TriTrypDB-45_T.bruceiLister427_2018.gff were downloaded from TriTrypDB and used as the references. The index of the reference genome was built using hisat2 2.1.0, and paired-end clean reads were aligned to the reference genome using HISAT2.

(iii) Quantification of gene expression levels. HTSeq v0.6.1 was used to calculate the number of reads mapped to each gene. The number of fragments per kilobase per million (FPKM) was calculated for each gene on the basis of the length of the gene and the number of reads mapped to the gene.

(iv) Differential expression analysis. Differential expression analysis of two conditions/group (three biological replicates per condition) was performed using the DESeq R package (1.18.0). The DESeq R package provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg approach for controlling the false-discovery rate. Genes with an adjusted P value of <0.05 found by DESeq were assigned as differentially expressed.

Data availability. The RNA-seq data set determined in this work has been submitted to NCBI GEO under accession number GSE143456.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 1.9 MB.
FIG S2, EPS file, 2.6 MB.
FIG S3, PDF file, 1.1 MB.
FIG S4, EPS file, 1.1 MB.
FIG S5, EPS file, 1.2 MB.
FIG S6, EPS file, 1.8 MB.
TABLE S1, DOCX file, 0.01 MB.

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