DNA topoisomerase IIIβ promotes cyst generation by inducing cyst wall protein gene expression in *Giardia lamblia*

Chin-Hung Sun, Shih-Che Weng, Jui-Hsuan Wu, Szu-Yu Tung, Li-Hsin Su, Meng-Hsuan Lin and Gilbert Aaron Lee

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Review timeline
Original submission: 19 September 2019
Revised submission: 28 November 2019
Final acceptance: 6 January 2020
Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOB-19-0228.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Do you have any ethical concerns with this paper?
No

Comments to the Author
Sun et al explore the role of TOP3 β in regulating Giardia differentiation. They demonstrate that TOP3 β localizes to the nucleus and binds to AT rich promoter sequences. They also show that over expression of this enzyme leads to up-regulation of CWP proteins and Myb2. Conversely knockdown with CRISRP based allele deletion results in reduced CWP and Myb2 expression. Overall the results are clearly presented. I reviewed an earlier version of this paper and the authors addressed my major criticisms, so I recommend acceptance with some minor revisions I will outline below.
Major Points
1. The change in localization observed for TOP3β in non-encysting cells (Fig 1) versus encysting cells in Sup Fig 3 is fascinating and this deserves more attention. That TOP3β localization changes from perinuclear to dispersed in the nucleus and is an important point that supports the authors assertion that TOP3β is involved in regulating differentiation. This same re-localization is not observed in Fig 1 perhaps because the cell is not at the right stage of encystation or is not actually encysting (no CWP1 staining here). The cell shown in Fig S3 is sort of mid-encystation based on the CWP1 staining. So I also wonder if the nuclear distribution changes with stage in the encystation response. This is potentially a really nice observation that if moved to Fig 1 will strengthen the paper because it shows TOP3β is specifically changing localization in actively differentiating cells. I would like the authors to include this result in Fig 1 and also quantify the number of cells that have dispersed nuclear versus perinuclear localization during encystation. It is also interesting that the mutants are more clearly nuclear localized during encystation. Since there is no quantification of this I don’t know if this is due to selected images or a real response to encystation stimulus.

2. The super-shift assay in Fig 5C is not very convincing. A similar experiment in Fig S5B on the other hand is very clear. I believe the authors, but this experiment should be repeated and hopefully a clearer result can be obtained. What also doesn’t make sense is that in S5B there are two bands one that is found in every lane and then a unique shifted band near the top of the blot in the presence of the antibody. In Fig 5C the same band is observed in every well although it does appear darker in the lane where the antibody was labeled. As an alternative to repeating the blot, the authors could quantify the intensity of this band versus the same position in the control lanes to convince us that there is enrichment in their replicates.

3. Regarding the CRISPR experiment I question the value of using CRISPR/CAS9 over homologous recombination. I am under the impression that CRISPR/CAS9 is not actually providing any benefit and that the authors are observing homologous recombination. After transfection we expect the provided repair template to enter one of the two nuclei based on Poxleitner et al 2008 and Carpenter et al 2012. Therefore, a single transfection should be able to edit two copies of the genome. It is not expected to generate a true knockout due to the two nuclei challenge. Here however, PCR (8B) and western blotting (8E) both point toward just a single allele being disrupted since genomic DNA and protein levels are pretty close to the 25% reduction level of losing a single allele. The authors mention the use of NHEJ inhibiting drugs which seem to be doing nothing since Ebner et al showed that homologous recombination can be used to knockout genes in Giardia. I wonder why the authors bother to invoke the use of CRISPR since there is no perceivable benefit? Note that I do not dispute their mutant has a phenotype. My concern is that their pseudo use of CRISPR/CAS9 will confuse readers.

Minor Points
1. Fig S1 is corrupted or there was an error in its construction. The region around T328 is not shown and neither is the red arrow that is mentioned by the legend. Please fix this.

2. Sentence on Line 157 is confusing. It reads as if it is interesting that ESVs contain CWP1. To me what is interesting is that the localization of TOP3β is more dispersed in this encysting cell.

3. Sentence on 342 is awkward please re-write

4. On 411 change catalytic to catalytically

5. I would change the last sentence of the conclusion. I don’t believe this study told us much about the evolution of TOP3β and whether TOP3β is really a viable drug target. The function of the gene wasn’t shown to be essential since only a single allele of TOP3β was deleted according to the PCR in Fig 8B which roughly corresponds to the 30% reduction in TOP3β protein levels. My recommendation: “Our study provides evidence for the important role of TOP3β in the
differentiation of Giardia trophozoites into cysts, leading to greater understanding of the mechanism regulating cell differentiation and parasite transmission.”

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have submitted a detailed and interesting study of the effect of DNA topoisomerase 3b (type 1) on encystation in Giardia. The title focuses on the effect of cyst generation related to cwp expression. However, I’m not convinced that there is enough causal information to warrant the use of “by inducing” in the title.

They have used transfection studies to study the effect of upregulation of TOP3b on expression of the cwps and myb2 on encystation. They have also used CRISPR/Cas9 to reduce expression of top3b and shown decreases in the above. (Of note, knockout is impossible with Giardia because of its polyploidy, so these approaches are reasonable).

Overall, the scientific component of the work seems solid, but I do have some questions regarding the data to be addressed by the authors:

Thus, I will begin with the results section:
For figure 1 and other figures, the authors simply use p<0.05, but unless <0.001, it would be helpful to see the numbers for CI and actual p value.
For Fig 4A, the difference between top3b positive and negative for linear DNA is not very convincing. Can the authors provide quantitative scan data from several runs?
Fig 5C, the mobility shifted lanes are not very convincing.

The bigger issues for correction relate to the writing:

1. The introduction has far too much of results in it. Almost 1/3 of the intro is results.
2. The biggest problem is the discussion. I felt like I was reading the results again when reading the discussion. There are two things that would greatly help. First, rather than reiterating results, explain the difficult parts and show how the results fit into other literature. Second, a figure with a model of what the authors think is happening would be very useful.
3. Although the writing is reasonably good, there are still occasional grammatical errors. If the journal has in-house copy editing, it should be ok.
4. A structured abstract would be nice if it fits with journal policy.

Decision letter (RSOB-19-0228.R0)

12-Nov-2019

Dear Dr Sun,

We are writing to inform you that the Editor has reached a decision on your manuscript RSOB-
19-0228 entitled "DNA topoisomerase III $\beta$ promotes cyst generation by inducing cyst wall protein gene expression in Giardia lamblia", submitted to Open Biology.

As you will see from the reviewers’ comments below, there are a number of criticisms that prevent us from accepting your manuscript at this stage. The reviewers suggest, however, that a revised version could be acceptable, if you are able to address their concerns. If you think that you can deal satisfactorily with the reviewer’s suggestions, we would be pleased to consider a revised manuscript.

The revision will be re-reviewed, where possible, by the original referees. As such, please submit the revised version of your manuscript within four weeks. If you do not think you will be able to meet this date please let us know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, please revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, please respond to the comments made by the referee(s) and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referee(s).

Please see our detailed instructions for revision requirements https://royalsociety.org/journals/authors/author-guidelines/

Once again, thank you for submitting your manuscript to Open Biology, we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
The Open Biology Team
mailto: openbiology@royalsociety.org

Reviewer(s)’ Comments to Author(s):

Referee: 1
Comments to the Author(s)

Sun et al explore the role of TOP3 $\beta$ in regulating Giardia differentiation. They demonstrate that TOP3 $\beta$ localizes to the nucleus and binds to AT rich promoter sequences. They also show that over expression of this enzyme leads to up-regulation of CWP proteins and Myb2. Conversely knockdown with CRISRP based allele deletion results in reduced CWP and Myb2 expression. Overall the results are clearly presented. I reviewed an earlier version of this paper and the authors addressed my major criticisms, so I recommend acceptance with some minor revisions I will outline below.

Major Points
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authors assertion that TOP3 β is involved in regulating differentiation. This same re-localization is not observed in Fig 1 perhaps because the cell is not at the right stage of encystation or is not actually encysting (no CWPI1 staining here). The cell shown in Fig S3 is sort of mid-encystation based on the CWPI1 staining. So I also wonder if the nuclear distribution changes with stage in the encystation response. This is potentially a really nice observation that if moved to Fig 1 will strengthen the paper because it shows TOP3 β is specifically changing localization in actively differentiating cells. I would like the authors to include this result in Fig 1 and also quantify the number of cells that have dispersed nuclear versus perinuclear localization during encystation. It is also interesting that the mutants are more clearly nuclear localized during encystation. Since there is no quantification of this I don’t know if this is due to selected images or a real response to encystation stimulus.

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3. Regarding the CRISPR experiment I question the value of using CRISPR/CAS9 over homologous recombination. I am under the impression that CRISPR/CAS9 is not actually providing any benefit and that the authors are observing homologous recombination. After transfection we expect the provided repair template to enter one of the two nuclei based on Poxleitner et al 2008 and Carpenter et al 2012. Therefore, a single transfection should be able to edit two copies of the genome. It is not expected to generate a true knockout due to the two nuclei challenge. Here however, PCR (8B) and western blotting (8E) both point toward just a single allele being disrupted since genomic DNA and protein levels are pretty close to the 25% reduction level of losing a single allele. The authors mention the use of NHEJ inhibiting drugs which seem to be doing nothing since Ebner et al showed that homologous recombination can be used to knockout genes in Giardia. I wonder why the authors bother to invoke the use of CRISPR since there is no perceivable benefit? Note that I do not dispute their mutant has a phenotype. My concern is that their pseudo use of CRISPR/CAS9 will confuse readers.

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3. Although the writing is reasonably good, there are still occasional grammatical errors. If the journal has in-house copy editing, it should be ok.
4. A structured abstract would be nice if it fits with journal policy.

Author’s Response to Decision Letter for (RSOB-19-0228.R0)
See Appendix A.

RSOB-19-0228.R1 (Revision)

Review form: Reviewer 1

Recommendation
Accept as is

Do you have any ethical concerns with this paper?
No
Comments to the Author
The authors have sufficiently addressed my concerns.

Decision letter (RSOB-19-0228.R1)

06-Jan-2020

Dear Dr SUN

We are pleased to inform you that your manuscript entitled "DNA topoisomerase III β promotes cyst generation by inducing cyst wall protein gene expression in Giardia lamblia" has been accepted by the Editor for publication in Open Biology.

If applicable, please find the referee comments below. No further changes are recommended.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it within the next 10 working days. Please let us know if you are likely to be away from e-mail contact during this time.

Thank you for your fine contribution. On behalf of the Editors of Open Biology, we look forward to your continued contributions to the journal.

Sincerely,
The Open Biology Team
mailto: openbiology@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
The authors have sufficiently addressed my concerns.
Dear Journal editor,

Thank you very much for the critical review of our manuscript. We appreciate the comments and are submitting a manuscript revised according to the critiques and questions of the reviewers. The responses and the changes in the text are detailed below each critique. They are marked by //.

The changed manuscript with highlight is shown below.

We hope that you find the revised manuscript suitable for publication in Open Biology.

Thank you very much,

Sincerely,

Chin-Hung Sun

12-Nov-2019

Dear Dr Sun,

We are writing to inform you that the Editor has reached a decision on your manuscript RSOB-19-0228 entitled "DNA topoisomerase IIIβ promotes cyst generation by inducing cyst wall protein gene expression in Giardia lamblia", submitted to Open Biology.

As you will see from the reviewers’ comments below, there are a number of criticisms that prevent us from accepting your manuscript at this stage. The reviewers suggest, however, that a revised version could be acceptable, if you are able to address their concerns. If you think that you can deal satisfactorily with the reviewer’s suggestions, we would be pleased to consider a revised manuscript.

The revision will be re-reviewed, where possible, by the original referees. As such, please submit the revised version of your manuscript within four weeks. If you do not think you will be able to meet this date please let us know immediately.

Appendix A

Dear Journal editor,

Thank you very much for the critical review of our manuscript. We appreciate the comments and are submitting a manuscript revised according to the critiques and questions of the reviewers. The responses and the changes in the text are detailed below each critique. They are marked by //.

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To revise your manuscript, log into https://mc.manuscriptcentral.com/rsob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, please revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, please respond to the comments made by the referee(s) and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referee(s).

Please see our detailed instructions for revision requirements https://royalsociety.org/journals/authors/author-guidelines/

Once again, thank you for submitting your manuscript to Open Biology, we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,

The Open Biology Team
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Reviewer(s)' Comments to Author(s):

Referee: 1
Comments to the Author(s)

Sun et al explore the role of TOP3β in regulating Giardia differentiation. They demonstrate that TOP3β localizes to the nucleus and binds to AT rich promoter sequences. They also show that over expression of this enzyme leads to up-regulation of CWP proteins and Myb2. Conversely
knockdown with CRISRP based allele deletion results in reduced CWP and Myb2 expression. Overall the results are clearly presented. I reviewed an earlier version of this paper and the authors addressed my major criticisms, so I recommend acceptance with some minor revisions I will outline below.

Major Points

1. The change in localization observed for TOP3β in non-encysting cells (Fig 1) versus encysting cells in Sup Fig 3 is fascinating and this deserves more attention.

// The mentioned Fig. 1 is Fig. 2B. In Fig. 2B-F, both the Veg (vegetative) and Enc (encyting) stages are shown. In Fig. S3B (finally moved to Fig. 2C), we repeated the Enc (encyting) part with CWP1 costaining and confirmed the same cell with expression of both TOP3β-HA and CWP1.

That TOP3β localization changes from perinuclear to dispersed in the nucleus and is an important point that supports the authors assertion that TOP3β is involved in regulating differentiation.

// The old Fig. S3B (finally moved to Fig. 2C) is a bit misleading, so we changed it to a new one. The localization of TOP3β is still in the nuclear periphery. In Fig. 2B, The same “nuclear periphery” localization of cells in both the Veg (vegetative) and Enc (encyting) stages was also shown.

// The perinuclear localization of the Giardia TOP3β may have its specific function. We wrote “Similarly, DNA topoisomerase I from yeast and human have been found to have a perinuclear distribution that may help function in DNA replication with perinuclear anchors of chromosomes (35).” (Line154)

This same re-localization is not observed in Fig 1 perhaps because the cell is not at the right stage of encystation or is not actually encysting (no CWP1 staining here).

// As we suggested above, there is no re-localization (we found the same “nuclear periphery” localization of TOP3β in Fig. 2B (including Veg and Enc) and Fig. S3B (finally moved to Fig. 2C)). The encysting cell with CWP1 staining is shown in Fig. S3B (finally moved to Fig. 2C).
The cell shown in Fig S3 is sort of mid-encystation based on the CWP1 staining.

We have changed the Fig S3B (finally moved to Fig. 2C). The encystation stage is based on the “positive” CWP1 staining as it is hard for us to classify the stage.

So I also wonder if the nuclear distribution changes with stage in the encystation response. This is potentially a really nice observation that if moved to Fig 1 will strengthen the paper because it shows TOP3β is specifically changing localization in actively differentiating cells.

We finally moved the Fig. S3B to Fig. 2C.

I would like the authors to include this result in Fig 1 and also quantify the number of cells that have dispersed nuclear versus perinuclear localization during encystation.

We observed the only one kind of localization of TOP3β: “nuclear periphery” in Fig. 2B (including Veg and Enc) and Fig. S3B (finally moved to Fig. 2C), so we did not quantify it.

It is also interesting that the mutants are more clearly nuclear localized during encystation. Since there is no quantification of this I don’t know if this is due to selected images or a real response to encystation stimulus.

As we mentioned in paper (lines 159-165), TOP3βm1 is also in nuclear periphery. TOP3βm2 and m3 have less nuclear peripheral staining. We found consistent staining results for the same staining pattern, so we did not quantify it.

2. The super-shift assay in Fig 5C is not very convincing. A similar experiment in Fig S5B on the other hand is very clear. I believe the authors, but this experiment should be repeated and hopefully a clearer result can be obtained.

We have changed the Fig. 5C with better image. This is the best gel from 3 same experiments. We have repeated the assays at least two times with similar results as shown in both Fig. 5C (in which double-stranded cwp1-45/-1 was used as the DNA probe) and Fig. S5B (in which single-stranded cwp3-30/+10 was used as the DNA probe). Since the double-stranded cwp1-45/-1 probe is different from the single-stranded cwp3-30/+10 probe in strand number, sequence, and
length, the bound forms for TOP3β are also different. The former are more indistinct (Fig. 5C, arrowheads). The supershifts in both Figures are quite obvious as indicated by arrows.

What also doesn’t make sense is that in S5B there are two bands one that is found in every lane and then a unique shifted band near the top of the blot in the presence of the antibody. In Fig 5C the same band is observed in every well although it does appear darker in the lane where the antibody was labeled. As an alternative to repeating the blot, the authors could quantify the intensity of this band versus the same position in the control lanes to convince us that there is enrichment in their replicates.

// In supershift assays, the anti-TOP3β was added in the reaction and the size of supershift complex (anti-TOP3β - TOP3β -DNA probe) will be bigger than the bound form (TOP3β - DNA probe) and may get stuck near the loading well (arrows in Fig. 5C and Fig. S5B). We quantified the region with arrows and showed the results in Fig. S5C. We added the text: “Both supershift results for the probes cwp1-45/-1 and cwp3 -30/+10F were significant as quantified in Fig. S5.” (page 13)

2. Regarding the CRISPR experiment I question the value of using CRISPR/CAS9 over homologous recombination. I am under the impression that CRISPR/CAS9 is not actually providing any benefit and that the authors are observing homologous recombination.

// We hope our thorough analysis can provide a better method for the protozoan Giardia, so we must do a lot of work on the CRISPR/Cas9 system using mlf genes as a model target gene (Lin et al., 2019)(ref 11). Due to the tetraploid genome in two nuclei of Giardia, it could be hard to disrupt a gene completely in Giardia. We only generated knockdown but not knockout mutants. The potential of CRISPR/Cas9 system to complete knockout genes of interest in Giardia awaits further studies to explore. Using a strong promoter to drive the expression of cas9 gene could improve knockout efficiency.

// The good part for our CRISPR/Cas9 system is: Because of the integration of pac gene in genomic DNA, the knockdown effect may last very long time. We also show the similar knockdown effect after removal of puromycin for a month (Lin et al., 2019)(ref 11).

// A CRISPRi system for stable transcriptional repression of a target gene in Giardia also has been developed (McInally SG, Hagen KD, Nosala C, Williams J, Nguyen K, Booker J, et al. Robust
and stable transcriptional repression in Giardia using CRISPRi. Mol Biol Cell. 2018 Oct 31:mbcE18090605.). We added this ref in our CRISPR paper (Lin et al., 2019)(ref 11).

After transfection we expect the provided repair template to enter one of the two nuclei based on Poxleitner et al 2008 and Carpenter et al 2012. Therefore, a single transfection should be able to edit two copies of the genome. It is not expected to generate a true knockout due to the two nuclei challenge. Here however, PCR (8B) and western blotting (8E) both point toward just a single allele being disrupted since genomic DNA and protein levels are pretty close to the 25% reduction level of losing a single allele.

The results from papers “Poxleitner et al 2008 and Carpenter et al 2012” are for analyzing genetic exchange occurs in cysts, not in trophozoites. For that study, they wanted to ensure the presence of only a single integrated construct in a trophozoite before encysting experiment. They tested clonal populations with PCR, and tested for the absence of episomes by PCR, and analyzed with fluorescence in situ hybridization (FISH). Therefore, they found a single nuclei staining.

In our TOP3β paper, our aim is to disrupt the top3β gene for functional analysis. We did not make clonal populations. We did not ensure the presence of only a single integrated construct in a trophozoite. We did not ensure the absence of episomes. Therefore, there could be some mixtures of successful and unsuccessful cells. Interestingly we still got 23% disruption efficiency.

The authors mention the use of NHEJ inhibiting drugs which seem to be doing nothing since Ebneter et al showed that homologous recombination can be used to knockout genes in Giardia.

Cre-lox method in Ebneter et al 2016 is also a very good method. We don’t have a chance to work on Cre-lox. We wrote in our CRISPR paper (Lin et al., 2019)(ref 11): “A Cre/loxP system allows persistence of gene disruption in the absence of drug selection in Giardia (Wampfler et al., 2014; Ebneter et al., 2016). It is interesting to compare CRISPR/Cas9 and Cre-lox systems in Giardia.”

I wonder why the authors bother to invoke the use of CRISPR since there is no perceivable benefit? Note that I do not dispute their mutant has a phenotype. My concern is that their pseudo use of CRISPR/CAS9 will confuse readers.
As we wrote above, there is always a need for gene disruption techniques in Giardia. That is why CRISPRi has also been developed. For our lab, disruption of top3β gene is the second successful example (the first one is mlf gene (Lin et al., 2019)(ref 11)). We wanted to check out how many genes can be successfully target-disrupted by CRISPR/Cas9.

Minor Points
1. Fig S1 is corrupted or there was an error in its construction. The region around T328 is not shown and neither is the red arrow that is mentioned by the legend. Please fix this.

   // We have changed Fig. S1.

2. Sentence on Line 157 is confusing. It reads as if it is interesting that ESVs contain CWP1. To me what is interesting is that the localization of TOP3β is more dispersed in this encysting cell.

   // We tried to emphasize the role of TOP3β by writting “Interestingly, the CWP1 protein was stained in the ESVs of TOP3β-HA positive stained cells (Fig. 2C), suggesting that TOP3β may function in inducing the ESV and thereby in inducing cyst formation.” As we mentioned above, the old Fig. S3B (finally moved to Fig. 2C) is a bit misleading, so we changed it to a new one. The localization of TOP3β is still in the nuclear periphery. In Fig. 2B, The same “nuclear periphery” localization of cells in both the Veg (vegetative) and Enc (encytong) stages was also observed.

3. Sentence on 342 is awkward please re-write

   // We have changed it. (line 342)

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function of the gene wasn’t shown to be essential since only a single allele of TOP3β was deleted according to the PCR in Fig 8B which roughly corresponds to the 30% reduction in TOPO3β protein levels. My recommendation: “Our study provides evidence for the important role of TOP3β in the differentiation of Giardia trophozoites into cysts, leading to greater understanding of the mechanism regulating cell differentiation and parasite transmission.”

// Many thanks for the suggestion. We have changed it. (lane 462)

Referee: 2
Comments to the Author(s)

The authors have submitted a detailed and interesting study of the effect of DNA topoisomerase 3b (type 1) on encystation in Giardia. The title focuses on the effect of cyst generation related to cwp expression. However, I’m not convinced that there is enough causal information to warrant the use of “by inducing” in the title.

They have used transfection studies to study the effect of upregulation of TOP3b on expression of the cwps and myb2 on encystation. They have also used CRISPR/Cas9 to reduce expression of top3b and shown decreases in the above. (Of note, knockout is impossible with Giardia because of its polyploidy, so these approaches are reasonable).

Overall, the scientific component of the work seems solid, but I do have some questions regarding the data to be addressed by the authors:

Thus, I will begin with the results section:
For figure 1 and other figures, the authors simply use p<0.05, but unless <0.001, it would be helpful to see the numbers for CI and actual p value.

// We have added p value and CI (error bars) in every figure.

For Fig 4A, the difference between top3b positive and negative for linear DNA is not very
convincing. Can the authors provide quantitative scan data from several runs?

// We have added quantitation data for the linear DNA bands. (Fig. 4A)

Fig 5C, the mobility shifted lanes are not very convincing.

// We have changed the Fig. 5C with better image. This is the best gel from 3 same experiments.

The bigger issues for correction relate to the writing:

1. The introduction has far too much of results in it. Almost 1/3 of the intro is results.

// We have cut it down. (page 5)

2. The biggest problem is the discussion. I felt like I was reading the results again when reading the discussion. There are two things that would greatly help. First, rather than reiterating results, explain the difficult parts and show how the results fit into other literature. Second, a figure with a model of what the authors think is happening would be very useful.

// We have cut it down. (Discussion part)
// We have added a model in Fig. 9. (for text, please see page 17)

3. Although the writing is reasonably good, there are still occasional grammatical errors. If the journal has in-house copy editing, it should be ok.

// We have checked the correctness of the grammar.

4. A structured abstract would be nice if it fits with journal policy.

// We have checked the Journal policy. It does not require structured abstract, so we did not change the abstract.
DNA topoisomerase IIIβ promotes cyst generation by inducing cyst wall protein gene expression in *Giardia lamblia*

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Abstract

*Giardia lamblia* causes waterborne diarrhea by transmission of infective cysts. Three cyst wall proteins (CWP) are highly expressed in a concerted manner during encystation of trophozoites into cysts. However, their gene regulatory mechanism is still largely unknown. DNA topoisomerases control topological homeostasis of genomic DNA during replication, transcription, and chromosome segregation. They are involved in a variety of cellular processes including cell cycle, cell proliferation, and differentiation, so they may be valuable drug targets. *G. lamblia* possesses a type IA DNA topoisomerase (TOP3β) with similarity to the mammalian topoisomerase IIIβ. We found that TOP3β was up-regulated during encystation and it possessed DNA-binding and cleavage activity. TOP3β can bind to the *cwp* promoters *in vivo* using norfloxacin-mediated topoisomerase immunoprecipitation assays. We also found TOP3β can interact with MYB2, a transcription factor involved in the coordinate expression of *cwp1-3* genes during encystation. Interestingly, overexpression of TOP3β increased expression of *cwp1-3* and *myb2* genes and cyst formation. Microarray analysis confirmed up-regulation of *cwp1-3* and *myb2* genes by TOP3β. Mutation of the catalytically important Tyr residue, deletion of C-terminal zinc ribbon domain or further deletion of partial catalytic core domain reduced the levels of cleavage activity, *cwp1-3* and *myb2* gene expression, and cyst formation. Interestingly, some of these mutant proteins were mis-localized to cytoplasm. Using a CRISPR/Cas9 system for targeted disruption of *top3β* gene, we found a significant decrease in *cwp1-3* and *myb2* gene expression and cyst number. Our results suggest that TOP3β may be functionally conserved, and involved in inducing *Giardia* cyst formation.
Introduction

*Giardia lamblia* is a frequent cause of waterborne diarrheal diseases in developing countries and in tourists (1, 2). After acute giardiasis, a higher risk of post-infectious irritable bowel syndrome has been reported (3). Children with chronic giardiasis are vulnerable to malnutrition due to malabsorption, resulting in delayed growth and mental development (4). A parasitic trophozoite is capable of transforming into a dormant cyst form, in which the cyst wall is essential for transmission of giardiasis during survival in fresh water or the new host’s stomach (1).

The small genome suggests *Giardia* as a simplified life form of evolutionary interest (5). It contains most pathways for life event but with fewer conserved components as compared with yeast (5). *Giardia* is also a good model for studying single cell differentiation as its easy transition between the trophozoite and cyst forms *in vitro* (1, 2). After sensing encystation stimuli, trophozoites perform coordinated synthesis of the three cyst wall proteins (CWPs) which are transported through encystation secretory vesicles (ESVs) to form a protective cyst wall (1, 2).

Signaling molecules and transcription factors, including CDK2, MYB2 (Myb1-like protein in the *Giardia* genome database), WRKY, PAX1, and E2F1, may play a role in inducing the *cwp* gene expression (6, 7, 8, 9, 10). We also found that a myeloid leukemia factor (MLF) protein plays an important role in inducing *Giardia* differentiation into cysts (11). We used our newly developed CRISPR/Cas9 system in *G. lamblia* for targeted disruption of *mlf* gene expression to analyze MLF (11).

Topoisomerases are essential enzymes that can overcome the topological problems of
chromosomes during DNA replication, transcription, recombination, and mitosis (12,13). They are involved in cell growth, tissue development, and cell differentiation (12,13,14). The type I topoisomerases function by cutting one strand of DNA, but type II topoisomerases cut two strands of DNA (12,13). Therefore, the type I topoisomerases have a weaker relaxation effect than type II (15). Human topoisomerases IIIα (TOP3α) and IIIβ (TOP3β) belong to type IA family (16). The human type IA topoisomerases are monomeric and ATP independent (16). They create a transient single-stranded DNA break by transesterification of a catalytic Tyr of the cleavage domain and a phosphodiester bond of DNA and to form a covalent 5’ phosphotyrosyl complex with DNA (11,12). They further act by passing a single stand of DNA through the break to disentangle DNA (11,12). They prefer to relax negative supercoiled DNA (16). The N-terminal Toprim domains of bacterial type IA topoisomerases forms active site region with domain 3, which contains catalytic Tyr residue (17). The C-terminal zinc ribbon domains of bacterial type IA topoisomerases binds to DNA and interacts with other proteins to unwind DNA (18).

Disruption of yeast topoisomerase III resulted in a significant growth defect (19). Topoisomerase IIIβ null mutant mouse had a shorter life span and spleen hypertrophy (20,21). Disruption of topoisomerase III gene from zebra fish can affect T cell differentiation (22). Human type IA topoisomerases are not drug targets, but all other human topoisomerases are important targets for cancer chemotherapy (23). Many anti-cancer compounds act through inhibiting topoisomerase activity in cancer cells (24). Many antibiotics can inhibit type II topoisomerase by stabilizing covalent topoisomerase-DNA cleavage complexes, including norfloxacin (25,26).

During *Giardia* encystation, a trophozoite with 2 nuclei (4N) may differentiate into a cyst with 4 nuclei (16N) by DNA replication and homologous recombination may occur in the cyst.
nuclei (1,27). Because type I topoisomerases play a critical role in cell differentiation (20,21,22), we asked whether type I topoisomerases could be important for *Giardia* encystation. In our previous study, we found that a *Giardia* type II topoisomerase (TOPO II) is an important factor involved in inducing encystation and the TOPO II inhibitor, etoposide, can inhibit *Giardia* growth and encystation (28). In this study, we further tried to understand the role of a type IA topoisomerase, TOP3β. We found that the expression of the *Giardia* TOP3β protein increased during *Giardia* encystation. TOP3β had DNA-binding and cleavage activity, and overexpression of TOP3β increased expression of *cwp1-3* and *myb2* and cyst formation. We used an approach similar to chromatin immunoprecipitation (ChIP) assays, norfloxacin-mediated topoisomerase immunoprecipitation assays (29) to confirm the association of TOP3β with these gene promoters in vivo. Oligonucleotide microarrays confirmed the up-regulation of the *cwp1-3* and *myb2* genes and identified up-regulation of many genes in the TOP3β-overexpressing cell line. Using mutation analysis and CRISPR/Cas9 system, we found evidence of TOP3β in inducing *Giardia* encystation. We also tested the effect of a type IA topoisomerase inhibitor, norfloxacin, and found that it inhibited *Giardia* growth and cyst formation, and increased the formation of cleavage complex of TOP3β and DNA. Using mutation analysis and CRISPR/Cas9 system, we found evidence of TOP3β in inducing *Giardia* encystation. Our results provide insights into the role of TOP3β in activation of *cwp* genes during *Giardia* encystation and into the effect of the TOP3β inhibitor, norfloxacin, on *Giardia* cyst formation and growth.
Results

Identification and characterization of \textit{top3}\textbeta gene. Four putative homologues for topoisomerases have been found in the \textit{G. lamblia} genome database (28). One is Topo II topoisomerase (open reading frame, orf, 16975). Orfs 15190 and 7615 are annotated as topoisomerase III, which belongs to type IA topoisomerases. Sequence analysis suggests that orfs 15190 and 7615 are similar to human TOP3\textbeta and TOP3\textalpha, respectively (see below). The last putative topoisomerase homologue is annotated as spo11 Type II DNA topoisomerases VI subunit A. We focused on understanding the role of orf 15190 (topoisomerase III\textbeta, TOP3\textbeta) in \textit{Giardia}. The deduced \textit{Giardia} TOP3\textbeta protein contains 973 amino acids with a predicted molecular mass of \(~107.06\) kDa and a pI of 8.40. It has a Toprim domain (residues 2 to 145) and a DNA topoisomerase domain (residues 159 to 610) as predicted by Pfam (Fig. 1A)(http://pfam.sanger.ac.uk/)(30). Toprim domain is a conserved active region typically found in type IA and type II topoisomerases (17). A zinc ribbon domain is present in the C terminus of \textit{Giardia} TOP3\textbeta (residues 645 to 973)(Fig. S1). The C-terminal zinc ribbon domains of bacterial type IA topoisomerases are important for DNA binding and interaction of RNA polymerase (18, 31, 32). \textit{Giardia} TOP3\textbeta also has a conserved Tyr (residue 328), corresponding to the catalytically important Tyrosines of \textit{E. coli} topoisomerase I (residue 319) and human TOP3\textbeta (residue 336) (Fig. 1A; Fig. S1)(20, 33). \textit{E. coli} topoisomerase III has a unique insertion which is a decatenation loop, which is and not found in \textit{Giardia} and other eukaryotic TOP3 (Fig. S1)(34). The full-length of \textit{Giardia} TOP3\textbeta has 28.73\% identity and 41.32\% similarity to that of human TOP3\textbeta (Calculated from Fig. S1). A phylogenic tree obtained from the alignment of the
topoisomerase III proteins from various organisms revealed that *Giardia* TOP3β (15190) is similar to TOP3β from other organisms, and that *Giardia* TOP3α (7615) is similar to TOP3α from other organisms (Fig. S2).

### Encystation-induced expression of the top3β gene and perinuclear localization of the TOP3β protein.

RT-PCR and quantitative real-time PCR analysis showed that the *top3β* mRNA increased by ~1.75-fold in 24h encysting cells (Fig. 1B). Western blot analysis with anti-TOP3β antibody revealed that the TOP3β level significantly increased during encystation (Fig. 1C).

To determine the expression of TOP3β protein, we prepared construct pPTOP3β, in which the *top3β* gene is controlled by its own promoter and contains an HA epitope tag (~1kDa) at its C terminus (Fig. 1D), and stably transfected it into *Giardia*. Similar to the expression pattern of the endogenous TOP3β protein (Fig. 1C), the level of TOP3β with the HA tag significantly increased during encystation (Fig. 1E).

### Change of localization of the TOP3β mutants.

To further understand the function of *Giardia* TOP3β, we analyzed the effect of mutation of TOP3β. The type I topoisomerases use an important Tyr of the cleavage domain as the active-site residue to create a transient single-stranded DNA break by transesterification (13). We tried to understand whether Tyr 328 of TOP3β, which corresponds to Tyr 336 of the human TOP3β, is also important for its activity (Fig. 2A, Fig. S1). Interestingly, the wild-type TOP3β-HA was located to the nuclear periphery that partly overlapped with DAPI and slightly to the cytoplasm (Fig. 2B). The perinuclear staining pattern of TOP3β-HA is not the endoplasmic reticulum (ER) staining. The typical ER staining of *Giardia* as shown in BIP staining contains reticulum shape of interconnected network in the cytoplasm and slightly perinuclear staining that did not overlap with DAPI (Fig. S3). Similarly,
DNA topoisomerases I from yeast and human have been found to have a perinuclear distribution that may help function in DNA replication with perinuclear anchors of chromosomes (35). Interestingly, the CWP1 protein was stained in the ESVs of TOP3β-HA positive stained cells (Fig. S32C), suggesting that TOP3β may function in inducing the ESV and thereby in inducing cyst formation. We found that mutation of the Tyr 328 to Phe did not change the localization of TOP3β to the nuclear periphery (TOP3βm1, Fig. 2A, Fig. 2C2D). We also found that deletion of the C-terminal 332 amino acids corresponding to the zinc ribbon domain (residues 642-973, pPTOP3βm2, Fig. 2A, Fig. 2D2E) resulted in a decrease in perinuclear localization, but an increase in cytosolic localization. Deletion of the C-terminal 552 amino acids corresponding to the zinc ribbon domain and a part of topoisomerase domain (residues 422-973, pPTOP3βm3, Fig. 2A, Fig. 2E2F) also decreased perinuclear localization, but increased localization to cytosolic vesicles. The background staining was very low as observed with wild-type WB trophozoites (Fig. 2F2G). TOP3βm2 and TOP3βm3 have lower but still some ability to localize to nuclear periphery (Fig. 2D2E, Fig. 2E2F). The results suggest that the Zinc ribbon domain may play a partial role in the perinuclear localization.

Overexpression of TOP3β induced the expression of the cwp1-3 and myb2 genes. We further investigated the effect of the Giardia TOP3β on encystation. We found a significant increase in the CWP1 and MYB2 levels in the TOP3β-overexpressing cell line relative to the control cell line (Fig. 1D, Fig. 3A) (36). The mRNA expression of the endogenous top3β plus vector-expressed top3β significantly increased in the TOP3β-overexpressing cell line relative to the control cell line (Fig. 3B, Fig. 3C). The mRNA expression of cwp1-3 and myb2 also increased in the TOP3β-overexpressing cell line (Fig. 3B, Fig. 3C). In previous studies, we
obtained consistent cyst number data for *Giardia* growth stage due to spontaneous differentiation (37). We found that the cyst number significantly increased in the TOP3β-overexpressing cell line (Fig. 3D). Similar results were obtained during encystation (Fig. S3). These findings suggest that overexpression of TOP3β can increase expression of cwp1-3 and myb2 and cyst formation.

We further investigated the role of TOP3β by mutation analysis. We found that the levels of TOP3βm1 and TOP3βm2 were similar to that of wild-type TOP3β during vegetative growth, but TOP3βm3 was expressed at a lower level (Fig. 3A). We also found that the CWP1 level significantly decreased in the TOP3βm1- and TOP3βm3-expressing cell lines relative to the wild-type TOP3β-expressing cell line (Fig. 3A). The CWP1 level also significantly decreased in the TOP3βm2-expressing cell line, but with a lower effect than in the TOP3βm1- and TOP3βm3-expressing cell lines (Fig. 3A). We further analyzed whether the transcript levels were changed. As shown by RT-PCR analysis, the mRNA expression of top3βm2-HA and top3βm3-HA increased compared with that of wild-type top3β-HA during vegetative growth, but the mRNA expression of top3βm1-HA decreased (Fig. 3B). The mRNA expression of cwp1-3 and myb2 significantly decreased in the TOP3βm1-m3-expressing cell lines relative to the wild-type TOP3β-expressing cell line (Fig. 3B, Fig. 3C). The level of cyst formation significantly decreased in the TOP3βm1-m3-expressing cell lines relative to the wild-type TOP3β-expressing cell line (Fig. 3D). Similar results were obtained during encystation (Fig. S3). The findings suggest a decrease in encystation-inducing activity of TOP3βm1-m3.

Oligonucleotide microarray assays confirmed up-regulation of cwp1-3 and myb2 expression in the TOP3β-overexpressing cell line to ~1.5 to ~11.9-fold of the levels that in the control cell line
(Fig. 3E). The *run* mRNA expression in the TOP3β-overexpressing cell line slightly decreased (~0.8-fold) (Fig. 3E). We found that 93 and 40 genes were significantly up-regulated (≥2-fold) and down-regulated (≤1/2) (*p*<0.05) in the TOP3β-overexpressing cell line relative to the vector control, respectively (Table S1). The *top3β* mRNA expression increased by ~2.1-fold (*p*<0.05) in the TOP3β-overexpressing cell line (Fig. 3E).

**TOP3β has DNA cleavage activity.** The type I topoisomerases have ability to bind to and cleave single-stranded DNA (12, 13, 38). *Drosophila* TOP3β cleaves DNA by forming a covalent topoisomerase-DNA complex (39). To test DNA cleavage activity of TOP3β, we expressed TOP3β in *E. coli* and purified it to >95% homogeneity. We performed DNA cleavage assays with purified recombinant TOP3β and pBluescript SK(+) plasmid. As shown in Fig. 4A, TOP3β has DNA cleavage activity.

Norfloxacin, a type II topoisomerase inhibitor, also inhibits *E. coli* topoisomerase I (type IA) at higher concentrations, resulting in anti-bacteria activity (25). Norfloxacin can inhibit topoisomerases by stabilizing covalent topoisomerase-DNA cleavage complexes (26). To understand whether norfloxacin can inhibit *Giardia* TOP3β DNA cleavage activity, we also performed DNA cleavage assays with norfloxacin. As shown in Fig. 4B, the addition of norfloxacin increased the amount of linear DNA, suggesting that norfloxacin can stabilize the TOP3β-DNA cleavage complex. We also tried to understand whether the products are from the covalent TOP3β-DNA cleavage complex. In a normal condition of the cleavage assay, proteinase K was included to stop the reaction by removing TOP3β from the cleavage complex (Fig. 4C).

When proteinase K was not included, the TOP3β-DNA cleavage complex can not enter the gel (Fig. 4C), suggesting that TOP3β can form a cleavage complex with DNA. The results indicate
that TOP3β may function as a topoisomerase in *Giardia*.

**Norfloxacin has anti-*Giardia* effect.** Norfloxacin is an inhibitor of type IA and type II topoisomerases with anti-bacteria activity (25). We found that norfloxacin increased DNA cleavage activity of TOP3β, indicating that norfloxacin can trap the cleavage complex of TOP3β (Fig. 4B). We also found that treatment with norfloxacin significantly reduced *Giardia* trophozoites growth (Fig. 4D). The half-maximal inhibitory concentration (IC50) of norfloxacin on *Giardia* was 497µM (Fig. 4D). The addition of 497µM norfloxacin also decreased cyst formation by 67% (Fig. S3). The results from the topoisomerase inhibitor norfloxacin suggest that TOP3β may regulate *Giardia* growth and differentiation into cysts.

**TOP3β mutants have a lower cleavage activity.** To understand which regions are important for cleavage activity, the specific TOP3β mutants were expressed in *E. coli*, and purified (Fig. 5A), and tested by cleavage assays. We found a decrease in cleavage activity of TOP3βm1 (with a mutation of the catalytically important Tyr 328) and TOP3βm3 (with a deletion of C-terminal 552 amino acids) and slight decrease in cleavage activity of TOP3βm2 (with a deletion of the C-terminal 332 amino acids) (Fig. 2A, Fig. 5B).

**TOP3β has DNA-binding activity and its mutants have lower DNA-binding activity.** We further tested DNA-binding activity of TOP3β. Electrophoretic mobility shift assays were performed with the purified TOP3β protein and double-stranded DNA sequences from the 5′-flanking region of the *cwp* genes. Incubation of a labeled double-stranded DNA probe, cwp1-45/-1, with TOP3β resulted in the formation of retarded bands (Fig. 5C). The binding specificity was confirmed by competition and supershift assays (Fig. 5C). The formation of the shifted cwp1-45/-1 bands was competed by a 200-fold molar excess of unlabeled cwp1-45/-1,
but not by the same excess of a nonspecific competitor, 18S-30/-1 (Fig. 5C), suggesting that
TOP3β did not bind to GC rich sequence. The bound form on cwp1-45/-1 could be supershifted
by an anti-TOP3β antibody (Fig. 5C). The results suggest that *Giardia* TOP3β can bind to the
cwp1 promoter (-45/-1 region). TOP3β was also shown to bind to the cwp3 promoter,
cwp3-30/+10 (Fig. 5D). To understand which regions of TOP3β are important for DNA binding,
the specific mutants were tested for their DNA-binding activity. There was only a slight decrease
in the DNA-binding activity of TOP3βm1, but there was a far more decrease in the DNA-binding
activity of TOP3βm2 and TOP3βm3 (Fig. 5D).

TOP3β was also shown to bind to cwp1-90/-46, and within this region it weakly bound to the
5’-region (cwp1-90/-69), the middle region (cwp1-78/-58), or the 3’-region (cwp1-68/-46) (Fig.
6). We found that TOP3β bound strongly to the cwp2-60/-31 and cwp3-30/+10 probes, and
weakly to the cwp2-30/+8 and cwp3-60/-31 probes (Fig. 6), suggesting that TOP3β can bind to
other encystation-induced promoters, cwp2 and cwp3. TOP3β also bound to a well characterized
ran core AT-rich promoter, ran-51/-20 (40) and weakly to ran-30/-1 (Fig. 6). TOP3β did not bind
to the 18S-30/-1, and 18S-60/-31 probes, which do not contain AT-rich sequence (Fig. 6).

Interestingly, TOP3β also weakly bound to a poly(A) sequence and a poly(A) sequence with a T
or TT insertion (Fig. S4), indicating that the TOP3β binding sequence contains AT-rich
sequences. The results suggest that TOP3β can strongly bind to the cwp1-3 and ran AT-rich
promoter regions.

We also performed DNA-binding assays with single-stranded DNA probes. We found that
TOP3β can bind to the single-stranded DNA of cwp1-3 promoters (cwp1-45/-1F, cwp1-90/-46F,
cwp2-60/-31F, cwp3-30/+10F) (Fig. S5), but it can not bind to the single-stranded DNA of 18S
promoter (18S-30/-1F), which does not contain AT-rich sequence (Fig. S5). TOP3β bound to cwp3-30/+10F could be supershifted by an anti-TOP3β antibody (Fig. S5). Both supershift results for the probes cwp1-45/-1 and cwp3-30/+10F were significant as quantified in Fig. S5. The results suggest that Giardia TOP3β can bind to the single-stranded DNA of the cwp1-3 promoters.

Recruitment of TOP3β to the top3β, cwp1-3 and myb2 promoters. We further used norfloxacin-mediated topoisomerase immunoprecipitation assay (28, 29), a method similar to ChIP assays, to study the association of TOP3β with the specific promoters. The addition of norfloxacin may increase the cleavage complex formation and thereby could increase ChIP sensitivity (29). We found that TOP3β was associated with its own promoter and the cwp1-3, myb2, and ran promoters during encystation (Fig. 7A, Fig. 7B). However, TOP3β was not associated with the U6 snRNA promoter (transcribed by pol III), or 18S ribosomal RNA promoter (transcribed by pol I) which has no TOP3β binding site (Fig. 7A).

Interaction between MYB2 and TOP3β associated complexes. It is possible that TOP3β may regulate encystation-induced cwp genes by interacting with other transcription factors. We further tried to understand whether TOP3β can interact with the encystation-induced MYB2 transcription factor (7). We performed co-immunoprecipitation experiments using the TOP3β-overexpressing cell line. The TOP3β-HA protein (~108 kDa) was expressed in the pPTOP3β stable cell line but not in the control cell line (5’Δ5N-Pac) (Fig. 7C) as detected by anti-HA antibody in Western blots (Fig. 7C). Overexpression of TOP3β in the pPTOP3β cell line also can be confirmed by the anti-TOP3β antibody (Fig. 7C). We found that TOP3β overexpression resulted in an increase in the MYB2 level (Fig. 7C). However, the ISCS level
decreased by TOP3β overexpression (Fig. 7C). We lysed the cells and immunoprecipitated TOP3β-HA with anti-HA antibody. Western blots of immunoprecipitates probed with anti-HA and anti-MYB2 indicate that MYB2 co-precipitates with TOP3β-HA in the absence or presence of DNase (Fig. 7D). The anti-HA antibody did not immunoprecipitate TOP3β-HA and MYB2 in the control cell line (Fig. 7D), nor did it immunoprecipitate ISCS in the pPTOP3β cell line (Fig. 7D). Far Western blot analysis confirmed the interaction between the N-terminal region of MYB2 (MYB2-N) and TOP3β-HA (Fig. 7E). The results suggest an interaction between MYB2 and TOP3β in a complex.

Targeted disruption of the top3β gene reduced expression of cwp1-3 and myb2. To further understand the function of TOP3β, we analyzed the effect of top3β gene disruption. We developed a CRISPR/Cas9 system to disrupt the mlf gene (11). We further adapted this system to the study the role of TOP3β. The CRISPR/Cas9 constructs were transfected into G. lamblia and TOP3βtd stable transfectants were established under puromycin selection (Fig. 8A). Scr7, an inhibitor of NHEJ, was added in the first replenishment of puromycin containing medium to increase knock-in efficiency via homologous recombination (11). The replacement of the top3β gene with the puromycin acetyltransferase (pac) gene was confirmed by PCR and sequencing analysis of genomic DNA (Fig. 8B, Fig. 8C, Fig. S6). The results show a successful disruption of the top3β gene by about 23% and a partial replacement of the top3β gene with the pac gene (Fig. 8B, Fig. 8C). It has been shown that G418 has cytotoxicity on mammalian cells (41). The toxicity of G418 may be mediated by blocking polypeptide synthesis during translation elongation (41). Inhibition of protein synthesis further results in oxidative stress and cell death (41). G418 also inhibits the growth of Giardia and can be used to select transfected cells (37).
We used G418 to test the drug sensitivity of the TOP3βtd cell line and found that TOP3βtd cell line exhibited increased sensitivity to it compared to the control cell line (Fig. 8D). We also found that the level of cyst formation significantly decreased in the TOP3βtd cell line relative to the control cell line during vegetative growth (Fig. 8E).

Western blot analysis confirmed the decrease of the TOP3β protein level in the TOP3βtd cell line relative to the control cell line (Fig. 8F). We found that the CWP1 level also significantly decreased in the TOP3βtd cell line relative to the control cell line (Fig. 8F). We further found the mRNA expression of top3β, cwp1-3, or myb2 significantly decreased in the TOP3βtd cell line relative to the control cell line (Fig. 8G, Fig. S7). Similar results were obtained during encystation (Fig. S8). The findings suggest a decrease in expression of cwp1-3 and myb2, drug sensitivity, and cyst formation by targeted disruption of the top3β gene.

We further tried to analyze results without puromycin. After selection, puromycin was removed from the TOP3βtd cell line to obtain the TOP3βtd –pu cell line. We found a successful disruption of the top3β gene by about 31% and a partial replacement of the top3β gene with the pac gene (Fig. S9). The level of cyst formation significantly decreased in the TOP3βtd –pu cell line relative to the control –pu cell line (Fig. S9). The levels of CWP1 and top3β, cwp1-3, and myb2 mRNA also significantly decreased in the TOP3βtd –pu cell line relative to the control –pu cell line (Fig. S9). Similar results were obtained during encystation (Fig. S10). The findings suggest a decrease in expression of cwp1-3 and myb2 and cyst formation by targeted disruption of the top3β gene without puromycin.
Discussion

The type I topoisomerases are involved in cell growth, tissue development, and cell differentiation (19; 20; 21). In this study, we identified and characterized a type IA topoisomerase, TOP3β, from *Giardia*. TOP3β has DNA-binding and cleavage activity of topoisomerases (Fig. 4A, Fig. 5C), as its catalytically important domains and residues are conserved. This suggests that the type IA topoisomerases may have evolved before divergence of *Giardia* from the main eukaryotic line of descent. The presence of at least one type II topoisomerase and one type IA topoisomerase suggests that they play the necessary roles in different organisms (42). Similarly, *Giardia* also has one type II topoisomerase and two type IA topoisomerases (orfs 16975, 15190, and 7615)(28).

Although mammalian topoisomerases have been studied intensively (18; 43), information on how they function to transcriptional regulation is still emerging (44,45). The type IA topoisomerases from *E. coli* and yeast can interact with RecQ DNA helicases to unwind hemicatenane structures during DNA replication (46), or unwind holiday junction during repairing DNA breaks or during chromosome segregation (43, 47, 48). *Drosophila* type IB topoisomerases interacts with a splicing factor, SR protein, to regulate gene expression (13, 49). The C-terminal zinc ribbon domain of *E. coli* topoisomerase I (type IA) is important for interaction of RNA polymerase (18). It helps bring to the transcription site for relaxation reaction (18), suggesting an importance of type I topoisomerases in transcription. The type IA topoisomerases may bind to DNA, regulate chromatin open, and thereby activate gene expression (31, 32, 50). Both type I and type II topoisomerases are recruited to genomic loci with higher transcriptional activity (45, 51). Human TOP3B targeted transcription start sites and...
induced transcription by repressing R-loop structures that inhibited transcription at target gene promoters (52,53). We found that *Giardia* TOP3β may have a similar role in inducing transcription to help encystation. Our results show that *Giardia* TOP3β can bind to the specific sequences in the core AT-rich initiator promoter region of the genes encoding key components of the cyst wall, *cwp1-3* (Fig. 6, Fig. S4, Fig. 9). Similarly, *Drosophila* topoisomerase IIIβ prefers to bind to AT-rich DNA sequences (39). We hypothesize that TOP3β, MYB2, and other transcription factors, can bind to the AT-rich elements or the proximal upstream regions and form complexes (Fig. 9) (7). This interaction may recruit RNA polymerase II to activate *cwp1-3* transcription (Fig. 9). The overexpressed TOP3β increased the levels of CWP1 and MYB2 and *cwp1-3* and *myb2* mRNA and cyst formation (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D). Results from the CRISPR/Cas9 system suggest a decrease in expression of CWP1, *cwp1-3* and *myb2*, and cyst formation by targeted disruption of the *top3β* gene (Fig. 8E, Fig. 8F, Fig. 8G). Furthermore, the addition of an inhibitor of type IA topoisomerases, norfloxacin, inhibited cell growth and cyst formation (Fig. 4D, Fig. S3). The results suggest a positive role of TOP3β in inducing *cwp1-3* and *myb2* gene expression and *Giardia* encystation.

Many *Giardia* gene promoters have the AT-rich initiator elements responsible for promoter activity and transcription start site selection (40,57,58). We have identified several transcription factors involved in the transactivation of the *cwp* genes, and they can bind to the AT-rich elements or the proximal upstream regions of the *cwp* promoters (7, 8,9,10,59,60,61). It has been reported that type IA topoisomerases can bind to DNA and activate gene expression (31,32, 50). In this study, we found that TOP3β can also bind to the AT-rich elements of the *cwp* promoters *in vitro* (Fig. 6). Norfloxacin-mediated topoisomerase immunoprecipitation assays confirmed the...
association of TOP3β with its own promoter and the cwp1-3 and myb2 promoters but not with the U6 promoter in vivo (Fig. 7A, Fig. 7B). ChIP assays confirmed the binding of encystation-induced transcription factors E2F1 and MYB2 to the cwp and myb2 gene promoters previously (7,10). E2F1 and MYB2 may interact together to activate expression of the cwp genes (10). We also found that MYB2 is co-immunoprecipitated with TOP3β (Fig. 7D). Treatment with DNase did not prevent the immunoprecipitation of Myb2 with TOP3β (Fig. 7D), suggesting that the interaction depends on protein-protein interaction but not DNA. Far Western blot analysis, a non-antibody method, was further used to confirm this interaction between the MYB2-N and TOP3β-HA to avoid the nonspecific problem (Fig. 7E). The MYB2-N can be phosphorylated by CDK2, which is involved in inducing encystation (6). The interaction of TOP3β and MYB2, and other transcription factors binding to the AT-rich elements or the proximal upstream regions (7), may be required for promoter activity and accurate transcription start site selection. TOP3β can bind to AT-rich elements of both the constitutive ran gene and encystation-induced cwp genes (Fig. 6). However, overexpressed TOP3β can induce the CWP1 level but not the RAN level (Fig. 3A). This could be due to a lack of cooperation of the encystation-specific transcription factors to transactivate the constitutive ran gene. Similar results were found in studies of other transcription factors (7, 8,9,10,60,61). Interestingly, expression of all three TOP3β mutants (TOP3βm1-3) have led to less expression levels of CWP1, cyst formation, and cwp1-3 and myb2 mRNA relative to the wild-type TOP3β (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D). However, they still have led to more expression levels of CWP1, cyst formation, and cwp1-3 and myb2 mRNA relative to the vector control (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D). It is possible that the mutants that were relatively overexpressed, may still interact with transcription factors, such as Myb2, to
activate expression of the *cwp* genes.

We also found that the *top3β* promoter contains the MYB2 binding sequences (Fig. S10)(61), suggesting that *top3β* gene expression is up-regulated by MYB2 and that TOP3β might play a positive role in *Giardia* encystation. Since overexpressed TOP3β increased the MYB2 level (Fig. 3A), there is a positive regulation cycle between TOP3β and MYB2. The human *top3α* promoters also contain the binding sequence of YY1 and USF1 activators, which are important for cell growth and differentiation (62). The induction ability of the overexpressed TOP3β in *cwp* transcription was active in vegetative and encystation stages (Fig. 3 and Fig. S3). Similarly, Myb2 and Pax1 transcription factors in expression system can also induce *cwp* transcription in both stages (9,28). This suggests that the specific promoter or enhancer elements for *cwp* transcription may be active in both stages.

The DNA topoisomerase domain (residues 159 to 610) of *Giardia* TOP3β is near the central region (Fig. 1A). A zinc ribbon domain is located in the C terminus (residues 645 to 973)(Fig. S1). Studies suggest that the C-terminal zinc ribbon domains of *E. coli* topoisomerase I and *Drosophila* topoisomerase IIIα are important for DNA binding (31, 32). The C-terminal zinc ribbon domain of *E. coli* topoisomerase I interacts with RNA polymerase to help bring to the transcription site for relaxation reaction (18). *We found that Deletion of the C-terminal 332 amino acids (residues 642-973) corresponding to the zinc ribbon domain of TOP3β (residues 642-973) resulted in reduction in DNA-binding activity, but only slight decrease in cleavage activity (TOP3βm2) (Fig. 5D, Fig. S1), suggesting that the zinc ribbon domain is important for DNA-binding. Deletion of C-terminal 552 amino acids (residues 422-973) corresponding to the zinc ribbon domain and a part of topoisomerase domain of TOP3β (residues 422-973) resulted in*
reduction in the cleavage activity (TOP3βm3) (Fig. 1A, Fig. 5B), indicating that the
topo-isomerase domain is important for cleavage activity. We also found that a mutation of the
catalytically important Tyr 328 resulted in a decrease in cleavage activity (TOP3βm1) (Fig. 1A, Fig. 5B). Tyr327 of topoisomerase IIIβ in Leishmania donovani is also catalytically important (63). Interestingly, mutation of the Tyr 328 to Phe did not change its perinuclear localization in both vegetative and encysting cells (TOP3βm1) (Fig. 2C2D). Mutation of this important Tyr also resulted in a significant decrease in the levels of CWP1, cyst formation, and cwp1-3 and myb2 mRNA (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D, Fig. 5B, Fig. 5D), suggesting a correlation of DNA cleavage activity and in vivo function.

Typically, nuclear localization signal (NLS) is a region rich with basic amino acids. Two putative NLS motifs were predicted in TOP3β using the PSORT program (http://www.psort.org/), including RKHR at 970, and RRAAQPKRHGPRGRKH at 957. We also found that deletion of the C-terminal 332 (residues 642-973) or 552 amino acids (residues 422-973) resulted in a decrease but not complete loss of perinuclear localization (TOP3βm2 or m3) (Fig. 2D2E, Fig. 2E2F), suggesting that the C-terminal zinc ribbon domain may play a partial role in the perinuclear localization and that other NLS motifs may be present in TOP3β. Deletion of the C-terminal 332 amino acids (TOP3βm2) resulted in a significant decrease in the levels of CWP1, cyst formation, and cwp1-3 and myb2 mRNA (TOP3βm2) (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D), but the effect is lower than the TOP3βm1 and TOP3βm3. As Shown in Fig. 2D2E, TOP3βm2 still has some ability to localize to nuclear periphery. Interestingly, DNA cleavage activity was less affected in this mutant (TOP3βm2) (Fig. 5B), suggesting again a correlation of DNA cleavage activity and in vivo function. The results suggest that TOP3β may enhance the
encystation-induced expression of *cwp1-3* and *myb2* through its cleavage activity.

*Drosophila* topoisomerase IIIβ prefers to bind to AT-rich DNA sequences (39). We also found that *Giardia* TOP3β can bind to the AT-rich promoter elements of the *cwp* genes (Fig. 6), and that it may bind to and up-regulate the *cwp* gene promoters to induce *Giardia* encystation. We found that 93 and 40 genes were significantly up-regulated and down-regulated in the TOP3β-overexpressing cell line relative to the vector control, respectively (Table S1). In addition, targeted disruption of the *top3β* gene resulted in a decrease in expression of *cwp1-3* and *myb2*, and cyst formation using the CRISPR/Cas9 system (Fig. 8), suggesting TOP3β may induce *Giardia* encystation. We also found that chemosensitivity of the TOP3βtd cell line significantly increased by the addition of G418 (Fig. 8D), suggesting that TOP3β may affect many genes involved in cell growth to survive antibiotic stress.

Norfloxacin, which belongs to quinolones, is an inhibitor of the type II topoisomerases, including topoisomerase II (DNA gyrase) and topoisomerase IV (25), it also inhibits type IA topoisomerases at higher concentrations (25). Quinolones can stabilize the topoisomerase II-DNA complex and prevent religation of DNA, resulting in anti-topoisomerase activity (23, 26, 64). A model suggests that quinolones can form complexes with DNA and topoisomerase IV and create barriers to DNA replication (65). We found that the addition of norfloxacin increased the cleavage complex formation of *Giardia* TOP3β (Fig. 4B), and the addition of norfloxacin significantly decreased cell growth and cyst formation (Fig. 4D, Fig. S3). The IC50 of norfloxacin is 497μM, which is similar to the IC50 for mammalian cells (470μM for CT-26 cells) (66). It is much higher than the IC50 of norfloxacin against *E. coli* (0.3μM) and the IC50 for inhibiting *E. coli* topoisomerase I activity (135μM) (25). The difference of effective norfloxacin
concentrations between *Giardia* and *E. coli* could be due to the variability of the overall
sequences of *Giardia* TOP3β and *E. coli* topoisomerase I, in which the region is helpful for
designing therapeutic drugs. Our results suggest that norfloxacin is less effective than the
standard drug metronidazole, which has been used often in the treatment of *Giardia* infection
with an IC50 of 2.1µM (67,68).

In this study, we found that TOP3β can induce expression of CWP1 and MYB2 that are
involved in encystation of the protozoan *Giardia*, suggesting that TOP3β may be functionally
conserved and involved in regulation of gene expression and cell differentiation. Further work is
necessary to find the norfloxacin derivatives that inhibit *Giardia* cyst formation and growth
without harming human cells. Our study provides evidence for the important role of TOP3β in
the differentiation of *Giardia* trophozoites into cysts, leading to greater understanding of the
evolution of eukaryotic topoisomerases during cell differentiation and help develop
anti-protozoan agents, the mechanism regulating cell differentiation and parasite transmission.
**Experimental procedure**

**Materials and Methods**

*G. lamblia culture.* Trophozoites *G. lamblia culture.* Trophozoites of *G. lamblia* WB, clone C6 (see ATCC 50803)(obtained from ATCC), were cultured in modified TYI-S33 medium (69).

Encystation was performed as previously described (54). Briefly, trophozoites grown to late log phase in growth medium were harvested and encysted for 24h in TYI-S-33 medium containing 12.5 mg/ml bovine bile at pH 7.8 at a beginning density of $5 \times 10^5$ cells/ml. In experiments exposing *Giardia* vegetative trophozoites to norfloxacin, WB clone C6 trophozoites were cultured in growth medium at a beginning density of $5 \times 10^5$ cells/ml with 0, 100, 200, 300, 400, 500, 600, 700, 800, or 497 $\mu$M norfloxacin. In experiments exposing *Giardia* vegetative trophozoites to G418, TOP3βtd and control cell line were cultured in growth medium at a beginning density of $1 \times 10^6$ cells/ml with 518 $\mu$M G418.

Cyst count. Cyst count was performed on the stationary phase cultures ($\sim 2 \times 10^6$ cells/ml) during vegetative growth as previously described (37). The cells were subcultured in growth medium with suitable selection drugs at an initial density of $1 \times 10^6$ cells/ml. Cells seeded at this density became confluent within 24h. Confluent cultures were maintained for an additional 8h to ensure that the cultures were in stationary phase (at a density of $\sim 2 \times 10^6$ cells/ml). Cyst count was performed on these stationary phase cultures. Cyst count was also performed on 24h encysting cultures. Total cysts including both type I and II cysts (70) were counted in a hemacytometer chamber.

Isolation and analysis of the top3β gene. Synthetic oligonucleotides used are shown in Table S2. The *G. lamblia* genome database (http://www.giardiadb.org/giardiadb/)(5,71) was
searched with the keyword “topoisomerase” for annotated genes. This search detected one putative homologue for topoisomerase IIIβ (TOP3β) (XM_001709742.1, orf 15190 in the G. lamblia genome database). The TOP3β coding region with 300 bp of 5′-flanking region was cloned and the nucleotide sequence was determined. To isolate the cDNA of the top3β gene, we performed RT-PCR with the top3β-specific primers using total RNA from G. lamblia. For RT-PCR, 5μg of DNase-treated total RNA from vegetative and 24h encysting cells was mixed with oligo (dT)12-18 and random hexamers and Superscript II RNase H- reverse transcriptase (Invitrogen). Synthesized cDNA was used as a template in subsequent PCR with primers top3βF and top3βR. Genomic and RT-PCR products were cloned into pGEM-T easy vector (Promega) and sequenced (Applied Biosystems, ABI). Comparison of genomic and cDNA sequences showed that the top3β gene contained no introns.

Genomic DNA extraction, PCR and quantitative real-time PCR analysis. Synthetic oligonucleotides used are shown in Table S2. Genomic DNA was isolated from trophozoites using standard procedures (Sambrook, 1989). For PCR, 250 ng of genomic DNA was used as a template in subsequent PCR. PCR analysis of top3β (XM_001709742.1, orf 15190), cwp1 (U09330, orf 5638), cwp2 (U28965, orf 5435), and ran (U02589, orf 15869) genes was performed using primers top3βF (PCR1F) and top3βR (PCR1R), PCR2F and PCR2R, cwp1F and cwp1R, cwp2F and cwp2R, ranF and ranR, respectively. For quantitative real-time PCR, SYBR Green PCR master mixture was used (Kapa Biosystems). PCR was performed using an Applied Biosystems PRISM™ 7900 Sequence Detection System (Applied Biosystems). Specific primers were designed for detection of the top3β, cwp1, cwp2, and ran genes: top3βrealF and top3βrealR; cwp1realF and cwp1realR; cwp2realF and cwp2realR; ranrealF and
ranrealR. Two independently generated stably transfected lines were made from each construct
and each of these cell lines was assayed three separate times. The results are expressed as a
relative expression level over control. Student’s t-tests were used to determine statistical
significance of differences between samples.

**RNA extraction, RT-PCR and quantitative real-time PCR analysis.** Synthetic
oligonucleotides used are shown in Table S2. Total RNA was extracted from *G. lamblia* cell line
during vegetative growth or encystation using TRIzol reagent (Invitrogen). For RT-PCR, 5µg of
DNase-treated total RNA was mixed with oligo (dT)12-18 and random hexamers and Superscript
II RNase H- reverse transcriptase (Invitrogen). Synthesized cDNA was used as a template in
subsequent PCR. Semi-quantitative RT-PCR analysis of top3β (XM_001709742.1, orf 15190),
top3β-ha, cwp1 (U09330, orf 5638), cwp2 (U28965, orf 5435), cwp3 (AY061927, orf 2421),
myb2 (AY082882, orf 8722), ran (U02589, orf 15869), and 18S ribosomal RNA (M54878, orf
t0019) gene expression was performed using primers top3β865F and top3β926R, top3β865F and
HAR, cwp1F and cwp1R, cwp2F and cwp2R, cwp3F and cwp3R, myb2F and myb2R, ranF and
ranR, 18SrealF and 18SrealR, respectively. For quantitative real-time PCR, SYBR Green PCR
master mixture was used (Kapa Biosystems). PCR was performed using an Applied Biosystems
PRISM 7900 Sequence Detection System (Applied Biosystems). Specific primers were
designed for detection of the top3β, top3β-ha, cwp1, cwp2, cwp3, myb2, ran, and 18S ribosomal
RNA genes: top3βrealF and top3βrealR; top3βHAF and HAR; cwp1realF and cwp1realR;
cwp2realF and cwp2realR; cwp3realF and cwp3realR; myb2realF and myb2realR; ranrealF and
ranrealR; 18SrealF and 18SrealR. Each primer pairs were determined for amplification
efficiency ~95% based on the slope of the standard curve. Two independently generated stably
transfected lines were made from each construct and each of these cell lines was assayed three
separate times. The results are expressed as a relative expression level over control. Student’s
\( t \)-tests were used to determine statistical significance of differences between samples.

**Plasmid construction.** Synthetic oligonucleotides used are shown in Table S2. All constructs
were verified by DNA sequencing with a BigDye Terminator 3.1 DNA Sequencing kit and an
Applied Biosystems 3100 DNA Analyzer (Applied Biosystems). Plasmid 5’Δ5N-Pac was a gift
from Dr. Steven Singer and Dr. Theodore Nash (Singer et al., 1998). Plasmid pgCas9 has been
previously described (11). To make construct pPTOP3\( \beta \), the \( \top 3\beta \) gene and its 300 bp of
5’-flanking region were amplified with oligonucleotides top3\( \beta \)NF and top3\( \beta \)MR, digested with
NheI and MluI, and cloned into NheI and MluI digested pPop2NHA (72). To make construct
pPTOP3\( \beta \)m1, the top3\( \beta \) gene was amplified using two primer pairs top3\( \beta \)m1F and top3\( \beta \)MR,
and top3\( \beta \)m1R and top3\( \beta \)NF. The two PCR products were purified and used as templates for a
second PCR. The second PCR also included primers top3\( \beta \)m1F and top3\( \beta \)MR, and the product
was digested with NheI and MluI and cloned into the NheI and MluI digested pPop2NHA (72).
To make construct pPTOP3\( \beta \)m2 or pPTOP3\( \beta \)m3, the top3\( \beta \) gene was amplified using primers
top3\( \beta \)NF and top3\( \beta \)m2MR or top3\( \beta \)m3MR, digested with NheI and MluI, and cloned into NheI
and MluI digested pPop2NHA (72).

The 620-bp 5’-flanking region of the top3\( \beta \) gene was amplified with oligonucleotides
top3\( \beta \)5HF and top3\( \beta \)5NR, digested with HindIII/NcoI and cloned into HindIII/NcoI digested
5’Δ5N-Pac, resulting in TOP3\( \beta \)5. The 700-bp 3’-flanking region of the top3\( \beta \) gene was amplified
with oligonucleotides top3\( \beta \)3XF and top3\( \beta \)3KR, digested with XbaI/KpnI and cloned into
XbaI/KpnI digested TOP3\( \beta \)5, resulting in TOP3\( \beta \)53. We used gene synthesis services from IDT.
to obtain the fragment top3β-guide. The NCBI Nucleotide Blast search was used to avoid the potential off-target effects of guide sequence. The top3β-guide was digested with KpnI/EcoRI and cloned into KpnI/EcoRI digested TOP3β53, resulting in pTOP3βtd.

**Transfection and Western blot analysis.** Cells transfected with the pP series plasmids containing the pac gene were selected and maintained with 54μg/ml (100μM) of puromycin as described (36,73). For CRISPR/Cas9 system, *Giardia* trophozoites were transfected with plasmids pTOP3βtd and pgCas9, and then selected in 100μM puromycin. The culture medium in the first replenishment contained 6μM Scr7 and 100μM puromycin. The TOP3βtd stable transfectants were established after selection. Stable transfectants were maintained at 100μM puromycin and were further analyzed by Western blotting, or DNA/RNA extraction. The replacement of the top3β gene with the pac gene was confirmed by PCR and sequencing. The control is *G. lamblia* trophozoites transfected with double amounts of 5’Δ5N-Pac plasmid and selected with puromycin. This kind of control was used because of its same puromycin condition and plasmid amounts. Puromycin can induce cwp expression (37). For the removal of puromycin experiments, puromycin was then removed from the medium for each stable cell line to obtain TOP3βtd –pu and control –pu cell lines. Subsequent analysis was performed after the removal of the drug for a month.

Western blots were probed with anti-V5-horseradish peroxidase (HRP) (Invitrogen), anti-HA monoclonal antibody (1/5000 in blocking buffer; Sigma), anti-TOP3β (1/10000 in blocking buffer) (see below), anti-CWP1 (1/10000 in blocking buffer) (7), anti-MYB2 (1/5000 in blocking buffer) (10), anti-α-tubulin (1/10000 in blocking buffer) (T6199, Sigma), anti-ISCS (1/10000 in blocking buffer), anti-BIP (1/10000 in blocking buffer), anti-RAN (1/10000 in blocking buffer)
(Chuang et al., 2012), or preimmune serum (1/5000 in blocking buffer), and detected with HRP-conjugated goat anti-mouse IgG (1/5000; Pierce) or HRP-conjugated goat anti-rabbit IgG (1/5000; Pierce) and enhanced chemiluminescence (ECL) (Millipore).

**Expression and purification of the recombinant TOP3β protein.** The genomic top3β, iscs, or bip gene was amplified using oligonucleotides top3βF and top3βR, iscsF and iscsR, or bipF and bipR, respectively. The product was cloned into the expression vector pET101/D-TOPO (Invitrogen) in frame with the C-terminal His and V5 tag to generate plasmid pTOP3β, pISCS, or pBIP. To make the pTOP3βm1, pTOP3βm2, or pTOP3βm3 expression vector, the top3β gene was amplified using primers top3βF and top3βR and specific template, including pPTOP3βm1, pPTOP3βm2 (use top3βm2R as reverse primer), or pPTOP3βm3 (use top3βm3R as reverse primer), and cloned into the expression vector. The pBIP, pTOP3β, pTOP3βm1, pTOP3βm2, or pTOP3βm3 plasmid was freshly transformed into *Escherichia coli* BL21 Star™(DE3) (Invitrogen). An overnight pre-culture was used to start a 250-ml culture. *E. coli* cells were grown to an A600 of 0.5, and then induced with 1mM isopropyl-D-thiogalactopyranoside (IPTG) (Promega) for 4h. Bacteria were harvested by centrifugation and sonicated in 10ml of buffer A (50mM sodium phosphate, pH 8.0, 300mM NaCl) containing 10 mM imidazole and protease inhibitor mixture (Sigma). The samples were centrifuged and the supernatant was mixed with 1 ml of a 50% slurry of nickel-nitrilotriacetic acid Superflow (Qiagen). The resin was washed with buffer A containing 20 mM imidazole and eluted with buffer A containing 250mM imidazole. Fractions containing BIP, TOP3β, TOP3βm1, TOP3βm2, or TOP3βm3 were pooled, dialyzed in 25 mM HEPES pH 7.9, 20 mM KCl, and 15% glycerol, and stored at -70°C. Protein purity and concentration were estimated by Coomassie Blue and silver staining compared with bovine...
serum albumin. BIP, TOP3β, TOP3βm1, TOP3βm2, or TOP3βm3 was purified to apparent
homogeneity (>95%).

For purification of ISCS, bacteria expressing pISCS were harvested by centrifugation and
sonicated in 10 ml of buffer G (100 mM sodium phosphate, 10mM Tris-Cl, 6M Guanidine
Hydrochloride, pH8.0) containing 10 mM imidazole and complete protease inhibitor cocktail
(Hydrochloride, pH8.0) containing 10 mM imidazole and complete protease inhibitor cocktail
of Ni-NTA superflow (Qiagen). The resin was washed with buffer B (100 mM sodium phosphate,
10mM Tris-Cl, 8M urea, pH8.0) and buffer C (100 mM sodium phosphate, 10mM Tris-Cl, 8M
urea, pH6.3) and eluted with buffer E (100 mM sodium phosphate, 10mM Tris-Cl, 8M urea,
pH4.5). Fractions containing ISCS were pooled, dialyzed in 25 mM HEPES pH 7.9, 40 mM KCl,
and 15 % glycerol, and stored at -70°C. Protein purity and concentration were estimated by
Coomassie Blue and silver staining compared with bovine serum albumin. ISCS protein was
purified to apparent homogeneity (>95%).

Generation of anti-TOP3β, anti-ISCS, or anti-BIP antibodies. Purified TOP3β, ISCS, or
BIP protein was used to generate rabbit polyclonal antibodies through a commercial vendor
(Angene, Taipei, Taiwan).

Immunofluorescence assay. The pPTOP3β, pPTOP3βm1, pPTOP3βm2, or pPTOP3βm3
stable transfectants were cultured in growth medium under puromycin selection. Cells cultured
in growth medium or encystation medium for 24h were harvested, washed in phosphate-buffered
saline (PBS), and attached to glass coverslips (2 × 10⁶ cells/coverslip) and then fixed and stained
(74). Cells were reacted with anti-HA monoclonal antibody (1/300 in blocking buffer; Sigma)
and anti-mouse ALEXA 488 (1/500 in blocking buffer, Molecular Probes) was used as the
detector. ProLong antifade kit with 4',6-diamidino-2-phenylindole (Invitrogen) was used for mounting. TOP3β, TOP3βm1, TOP3βm2, or TOP3βm3 was visualized using a Leica TCS SP5 spectral confocal system.

**Electrophoretic mobility shift assay.** Double-stranded oligonucleotides specified throughout were 5'-end-labeled as described (40). Binding reaction mixtures contained the components described (59). Labeled probe (0.02 pmol) was incubated for 15 min at room temperature with 5 ng of purified TOP3β, TOP3βm1, TOP3βm2, or TOP3βm3 protein in a 20μl volume supplemented with 0.5μg of poly (dl-dC) (Sigma). Competition reactions contained 200-fold molar excess of cold oligonucleotides. In an antibody supershift assay, 0.8μg of an anti-TOP3β antibody (see above) was added to the binding reaction mixture. The mixture was separated on a 6% acrylamide gel by electrophoresis.

**DNA Cleavage assays.** Cleavage assays were performed as described (75). Reaction was performed in a 25μl mixture containing 10mM Tris-HCl pH 7.5, 100mM KCl, 5mM MgCl2, 30μg/ml BSA, 300ng pBluescript SK(+) plasmid, and 10ng purified TOP3β, TOP3βm1, TOP3βm2, or TOP3βm3. Some reactions contained 4.8mM norfloxacin dissolved in Me2SO or Me2SO as a control to test the effect on cleavage activity of topoisomerases. After incubation at 37°C for 30min, reaction was stopped by addition of 0.5% SDS, 10mM EDTA, and 2μg/μl proteinase K and incubation at 37°C for 30min. The resulting DNA was separated by electrophoresis on 1 % agarose gels plus 25μg/ml ethidium bromide.

**Microarray analysis.** RNA was quantified by A260nm by an ND-1000 spectrophotometer (Nanodrop Technology, USA) and qualititated by a Bioanalyzer 2100 (Agilent Technology) with an RNA 6000 Nano LabChip kit. RNA from the pPTOP3β cell line was labeled by Cy5 and RNA
from the 5'△5N-Pac cell line was labeled by Cy3. 0.5μg of total RNA was amplified by a Low
RNA Input Quick-Amp labeling kit (Agilent Technologies) and labeled with Cy3 or Cy5 (CyDye,
Agilent Technologies) during the in vitro transcription process. 0.825μg of Cy-labeled cRNA was
fragmented to an average size of about 50-100 nucleotides by incubation with fragmentation
buffer at 60°C for 30 minutes. Correspondingly fragmented labeled cRNA was then pooled and
hybridized to a G. lamblia oligonucleotide microarray (Agilent Technologies, USA) at 65°C for
17h. After washing and drying by nitrogen gun blowing, microarrays were scanned with an
Agilent microarray scanner (Agilent Technologies) at 535 nm for Cy3 and 625 nm for Cy5.
Scanned images were analyzed by Feature Extraction version 10.5.1.1 software (Agilent
Technologies), and image analysis and normalization software was used to quantify signal and
background intensity for each feature; data were substantially normalized by the rank
consistency filtering LOWESS method. All data is MIAME compliant and that the raw data has
been deposited in a MIAME (http://www.mged.org/Workgroups/MIAME/miame.html)
compliant database (GEO) with accession number GSE109912.

Co-immunoprecipitation assay. The specific stable transfectants were cultured in encystation
medium with puromycin (5×10⁷ cells in 45 ml medium) and harvested after 24h in encystation
medium with puromycin and washed in phosphate-buffered saline. Cells were lysed in luciferase
lysis buffer (Promega) and protease inhibitor (Sigma) and then vortexed with glass beads. The
cell lysates were collected by centrifugation and then incubated with anti-HA antibody
conjugated to beads (Sigma). The beads were washed four times with luciferase lysis buffer
(Promega). Finally the beads were then resuspended in sample buffer and analyzed by Western
blotting and probed with anti-HA monoclonal antibody (1/5000 in blocking buffer; Sigma),
TOP3β (1/10000 in blocking buffer) (see above), anti-MYB2, or anti-ISCS (1/10000 in blocking buffer) (see above), and detected with HRP-conjugated goat anti-mouse IgG (Pierce, 1/5000) or HRP-conjugated goat anti-rabbit IgG (Pierce, 1/5000) and ECL (GE Healthcare).

**Far Western blot analysis.** The Myb2-N and ISCS with a V5-tag at C terminus were expressed in *E. coli* and purified as previously described (6) and as above, respectively. The MYB2-N-V5 and ISCS-V5 were resolved by SDS-PAGE, transferred onto PVDF membranes, refolded in renaturation buffers, and incubated with lysate from the pPTOP3β cell line as described in co-immunoprecipitation assays. Bound TOP3β-HA was detected with monoclonal anti-HA antibody (Sigma). The signal was detected with HRP-conjugated goat anti-mouse IgG (GE Healthcare) and ECL (GE Healthcare). Additional membranes for the resolved MYB2-N-V5 and ISCS-V5 proteins were incubated with anti-V5-HRP antibody and detected by ECL (GE Healthcare) to determine where the V5-tagged proteins migrated.

**Norflaxacin-mediated topoisomerase immunoprecipitation assays.** The WB clone C6 cells were inoculated into encystation medium containing 497μM norflaxacin (5×10^7 cells in 45 ml medium) and harvested after 24h and washed in phosphate-buffered saline. The assay was performed as previously described (28) with some modifications. Formaldehyde was then added to the cells in phosphate-buffered saline at a final concentration of 1%. Cells were incubated at room temperature for 15 min and reactions were stopped by incubation in 125 mM glycine for 5 min. After phosphate-buffered saline washes, cells were lysed in luciferase lysis buffer (Promega) and protease inhibitor (Sigma) and then vortexed with glass beads. The cell lysate was sonicated on ice with Biorupter Plus (Diagenode) and then centrifuged. Chromatin extract was incubated with protein G plus/protein A-agarose (Merck) for 1h. After the removal of protein G.
plus/protein A-agarose, the precleared lysates were incubated with 2μg of anti-TOP3β antibody or preimmune serum for 2h and then incubated with protein G plus/protein A-agarose (Merck) for 1h. The beads were washed with luciferase lysis buffer (Promega) twice and phosphate-buffered saline twice. The beads were resuspended in elution buffer containing 50 mM Tris-HCl, pH 8.0, 1% SDS and 10 mM EDTA at 65°C for 4h. To prepare DNA representing input DNA, 2.5% of precleared chromatin extract without incubation with anti-TOP3β was incubated with elution buffer at 65°C for 4h. Eluted DNA was purified by the QIAquick PCR purification kit (Qiagen). Purified DNA was subjected to PCR reaction followed by agarose gel electrophoresis. Primers 18S5F and 18S5R were used to amplify the 18S ribosomal RNA gene promoter as a control for our analysis. Primers top3β5F and top3β5R, cwp15F and cwp15R, cwp25F and cwp25R, cwp35F and cwp35R, myb25F and myb25R, and U65F and U65R, were used to amplify top3β, cwp1, cwp2, cwp3, myb2, and U6 gene promoters within the -200 to -1 region.
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**Figure legends**

Fig. 1. Analysis of top3β gene expression. (A) Schematic representation of the *Giardia* TOP3β protein. The green box and red box indicate the Toprim domain and Topoisom_bac domain, respectively, as predicted by pfam. The conserved Tyr 328 (Y328) is indicated. (B) RT-PCR and quantitative real-time PCR analysis of top3β gene expression. RNA samples were prepared from *G. lamblia* wild-type non-transfected WB cells cultured in growth (Veg, vegetative growth) or encystation medium and harvested at 24h (Enc, encystation). RT-PCR was performed using primers specific for top3β, cwp1, ran, and 18S ribosomal RNA (18S rRNA) genes, respectively (left panel). Real-time PCR was performed using primers specific for top3β and 18S ribosomal RNA genes, respectively (right panel). Transcript levels were normalized to 18S ribosomal RNA levels. —Fold changes in mRNA expression are shown as the ratio of transcript levels in encysting cells relative to vegetative cells. Results are expressed as the means ± 95% confidence intervals (error bars) of at least three separate experiments. *, *P* < 0.05 was considered significant and the value was shown. As controls, we found that the mRNA expression of cwp1 and ran significantly increased and decreased during encystation, respectively. (C) TOP3β level increased during encystation. The wild-type non-transfected WB cells were cultured in growth (Veg, vegetative growth) or encystation medium for 24h (Enc, encystation) and then subjected to SDS-PAGE and Western blot analysis. The blot was probed with anti-TOP3β and anti-α-tubulin antibodies, respectively. Equal amounts of protein loading were confirmed by SDS-PAGE and Coomassie Blue staining. The α-tubulin level slightly decreased during encystation. The intensity of bands from three Western blot assays was quantified using Image J. The ratio of TOP3β protein over the loading control (Coomassie Blue-stained proteins) is calculated. Fold
change is calculated as the ratio of the difference between the Enc sample and Veg sample, to which a value of 1 was assigned. Results are expressed as mean ± 95% confidence intervals, SD.

*P < 0.05* was considered significant and the value was shown. (D) Diagrams of the 5′Δ5N-Pac and pPTOP3β plasmid. The *pac* gene (open box) is under the control of the 5′- and 3′-flanking regions of the glutamate dehydrogenase (*gdh*) gene (striated box). In construct pPTOP3β, the *top3β* gene is under the control of its own 5′-flanking region (open box) and the 3′-flanking region of the *ran* gene (dotted box). The filled black box indicates the coding sequence of the HA epitope tag. (E) TOP3β-HA level increased during encystation in TOP3β-overexpressing cells. The pPTOP3β stable transfectants were cultured in growth (Veg, vegetative growth) or encystation medium for 24h (Enc, encystation) and then subjected to SDS-PAGE and Western blot analysis. The blot was probed with anti-HA and anti-RAN antibodies, respectively. Equal amounts of protein loading were confirmed by SDS-PAGE and Coomassie Blue staining. The RAN level slightly decreased during encystation. The ratio of TOP3β-HA protein over the loading control (Coomassie Blue-stained proteins) is calculated as described in Fig. 1C.

**Fig. 2.** Localization of TOP3β mutants. (A) Diagrams of TOP3β and TOP3βm1-3. The residue Tyr 328 (Y328), which is important for TOP3β activity, is mutated to Phe (F328) in TOP3βm1. TOP3βm2 remains the same as wild-type TOP3β, except that it does not contain the C-terminal zinc ribbon domain (deletion of residues 642–973). TOP3βm3 remains the same as wild-type TOP3β, except that it does not contain the C-terminal zinc ribbon domain and part of the Topoisom_bac domain (deletion of residues 422–973). The *top3β* gene was mutated and subcloned to replace the wild-type *top3β* gene in the backbone of pPTOP3β (Fig. 1D), and the
resulting plasmids pPTOP3βm1-3 were transfected into *Giardia*. The expression cassettes of the *pac* gene and *top3β* gene are the same as in Fig. 1D. (B) Perinuclear localization of the TOP3β protein. The pPTOP3β stable transfectants were cultured in growth (Veg, left panel) or encystment medium for 24h (Enc, right panel), and then subjected to immunofluorescence analysis using anti-HA antibody for detection. The upper panels show that the TOP3β protein is localized to the nuclear periphery and slightly to the cytoplasm of vegetative and encysting trophozoites. The middle panels show the DAPI staining of cell nuclei. The bottom panels show the merged images. Some perinuclear staining of TOP3β-HA overlapped with DAPI. (C) Localization of CWP1 in the TOP3β-overexpressing cell line. The pPTOP3β stable transfectants were cultured in encystation medium for 24h and then subjected to immunofluorescence assays. The endogenous CWP1 protein and vector-expressed TOP3β-HA protein were detected by anti-CWP1 and anti-HA antibodies, respectively. The left panel shows that the TOP3β-HA protein is localized to the nuclear periphery and slightly to the cytoplasm. The middle panel shows that the CWP1 protein is localized to the ESVs. The right panel shows the merged image. (CD) Immunofluorescence analysis of TOP3βm1-3 distribution. The pPTOP3βm1-3 stable transfectants were cultured and then subjected to immunofluorescence analysis as described in Fig. 2B. The products of pPTOP3βm1 localized to the nuclear periphery that overlapped with DAPI with slight cytoplasmic staining in both vegetative and encysting trophozoites (panel CD). The products of pPTOP3βm2 localized to the cytoplasm with minor presence in perinuclear region that overlapped with DAPI in both vegetative and encysting trophozoites (panel DE). The products of pPTOP3βm3 localized to the vesicles in cytoplasm with minor presence in perinuclear region that overlapped with DAPI in both vegetative and encysting trophozoites.
encysting trophozoites (panel EF). (FG) Negative control for immunofluorescence. The wild-type WB trophozoites were cultured in growth (Veg, vegetative growth) and then subjected to immunofluorescence analysis using anti-HA antibody for detection as described in Fig. 2B.

Fig. 3. Induction of cwp1-3 and myb2 gene expression in the TOP3β-overexpressing cell line. (A) Overexpression of TOP3β increased the CWP1 and MYB2 levels. The 5’Δ5N-Pac, pPTOP3β, pPTOP3βm1, pPTOP3βm2, and pPTOP3βm3 stable transfectants were cultured in growth medium and then subjected to SDS-PAGE and Western blot. The blot was probed with anti-HA, anti-CWP1, anti-MYB2, and anti-RAN antibodies, respectively. Equal amounts of protein loading were confirmed by SDS-PAGE and Coomassie Blue staining. A similar level of the RAN protein was detected. The intensity of bands from three Western blot assays was quantified using Image J. The ratio of CWP1 and MYB2 proteins over the loading control RAN is calculated. Fold change is calculated as the ratio of the difference between the specific cell line and 5’Δ5N-Pac cell line, to which a value of 1 was assigned. Results are expressed as mean ± 95% confidence intervals. SD. *P<0.05 was considered significant and the value was shown. (B) RT-PCR analysis of gene expression in the TOP3β- and TOP3β mutants-expressing cell lines. The 5’Δ5N-Pac, pPTOP3β, and pPTOP3βm1-m3 stable transfectants were cultured in growth medium and then subjected to RT-PCR analysis using primers specific for top3β-ha, top3β, cwp1, cwp2, cwp3, myb2, and 18S ribosomal RNA genes, respectively. Similar levels of the 18S ribosomal RNA for these samples were detected. (C) Quantitative real-time PCR analysis of gene expression in the TOP3β- and TOP3β mutants-expressing cell lines. Real-time PCR was performed using primers specific for top3β, cwp1, cwp2, myb2, and 18S ribosomal RNA genes,
respectively, as described in Fig. 1B. (D) TOP3β overexpression increased cyst formation. The
pPTOP3β and pPTOP3βm1-m3 stable transfectants were cultured in growth medium and then
subjected to cyst count as described under “Materials and Methods”. The sum of total cysts is
expressed as a relative expression level over control. Values are shown as means ± 95%
confidence intervals. *, P<0.05 was considered significant and the value was shown. (E)
Microarray analysis. Microarray data were obtained from the 5′Δ5N-Pac and pPTOP3β cell lines
during vegetative growth. Fold-changes are shown as the ratio of transcript levels in the
pPTOP3β cell line relative to the 5′Δ5N-Pac cell line. Results are expressed as the mean ± 95%
confidence intervals of at least three experiments. p<0.05 was considered significant and
the value was shown.

Fig. 4. DNA cleavage activity of TOP3β and effect of norfloxacin. (A) TOP3β has DNA
cleavage activity. DNA cleavage assays were performed with purified recombinant TOP3β and
pBluescript SK(+) plasmid (3.0 kb). Components in the reaction are indicated above the lanes.
Typically, 10 ng TOP3β was mixed with 300 ng plasmid DNA. Linearized plasmid was included
as a size marker. The intensity of linear DNA bands from three assays was quantified using
Image J. Fold change is calculated as the ratio of the “+ TOP3β” sample to the “- TOP3β”
sample, to which a value of 1 was assigned. Results are expressed as mean ± 95% confidence
intervals. p<0.05 was considered significant and the value was shown. (B) Norfloxacin increased
the cleavage complexes. DNA cleavage assays were performed with purified recombinant
TOP3β and pBluescript SK(+) plasmid. Norfloxacin was added in the reaction as indicated
above the lanes. Typically, 10 ng TOP3β was mixed with 300 ng plasmid DNA. Norfloxacin was
dissolved in Me2SO. Adding Me2SO to the reaction mix was used as a control (lane 3). Adding 4.8 mM norfloxacin to the reaction mix increased the TOP3β DNA cleavage complexes (lane 4). Linearized plasmid was included as a size marker. (C) TOP3β formed covalent complexes with DNA. DNA cleavage assays were performed with purified recombinant TOP3β and pBluescript SK(+) plasmid. Norfloxacin was added in the reaction as indicated above the lanes. After reaction, proteinase K at a final concentration of 2 μg/μl was added to the stop reaction of the cleavage assay, and then the products were analyzed by agarose gel electrophoresis. The same volume of ddH2O was used for a negative reaction. (D) Anti-Giardia activity of norfloxacin. The wild-type non-transfected WB cells were subcultured at an initial density of 5×10^4 cells/ml in growth medium containing 0, 100, 200, 300, 400, 500, 600, 700, or 800 μM norfloxacin for 24h and then subjected to cell count. An equal volume of Me2SO was added to cultures as a negative control. The sum of total cells is expressed as a relative expression level over control. Values are shown as means±95% confidence intervals±S.E. of three independent experiments.
translation start site of the cwp1 gene). Components in the binding reaction mixtures are indicated above the lanes. The arrowheads indicate the shifted complexes. The TOP3β-binding specificity was confirmed by competition and supershift assays. Some reaction mixtures contained 200-fold molar excess of cold oligonucleotides or 0.8μg of anti-TOP3β antibody as indicated above the lanes. The transcription start sites of the cwp1 and cwp3 genes are indicated by asterisks. The AT-rich initiator elements spanning the transcription start sites are underlined. (D) Decrease in DNA-binding activity of TOP3β mutants. Electrophoretic mobility shift assays were performed using purified TOP3β and its mutants and the 32P-end-labeled oligonucleotide probe cwp3-30/+10. The arrowheads indicate the shifted complexes. **Fig. 6.** Detection of TOP3β binding sites in multiple promoters. Electrophoretic mobility shift assays were performed using purified TOP3β and 32P labeled oligonucleotide probes. Components in the binding reaction mixtures are indicated above the lanes. The transcription start sites of the cwp1, cwp2, and cwp3 genes determined from 24-h encysting cells are indicated by asterisks. The AT-rich initiator elements spanning the transcription start sites are underlined. The translation start sites of the cwp2 and cwp3 genes are bold. “18S” represents 18S ribosomal RNA. **Fig. 7.** Recruitment of TOP3β to the cwp and myb2 promoters and interaction between TOP3β and MYB2. (A) ChIP analysis of recruitment of TOP3β to the cwp and myb2 promoters. The nontransfected WB cells were cultured in encystation medium containing 497μM norfloxacin for 24h and then subjected to norfloxacin-mediated topoisomerase immunoprecipitation assays.
Anti-TOP3β was used to assess binding of TOP3β to endogenous gene promoters. Preimmune serum was used as a negative control. Immunoprecipitated chromatin was analyzed by PCR using primers that amplify the 5′-flanking region of the specific genes. At least three independent experiments were performed. Representative results are shown. Immunoprecipitated products of TOP3β yield more PCR products of the top3β, cwp1, cwp2, cwp3, myb2 gene promoters, indicating that TOP3β bound to these promoters (+). However, the anti-TOP3β antibody did not enrich the U6 promoter fragment (-). The 18S ribosomal RNA gene promoter was used as a negative control (-). (B) ChIP analysis coupled by quantitative PCR. Values represented as a percentage of the antibody-enriched chromatin relative to the total input chromatin (% of Input). Results are expressed as the mean ± 95% confidence intervals of at least three experiments. p<0.05 was considered significant and the value was shown. (C) Expression of the TOP3β-HA, TOP3β, MYB2, and ISCS proteins detected in whole cell extracts for co-immunoprecipitation assays (Input). The 5′Δ5N-Pac and pPTOP3β stable transfectants were cultured in encystation medium for 24h and then subjected to SDS-PAGE and Western blot analysis as described in Fig. 3A. The blot was probed with anti-TOP3β, anti-MYB2, anti-ISCS, and anti-RAN antibodies, respectively. The intensity of bands from three Western blot assays was quantified as described in Fig. 3A. (D) Interaction between TOP3β and MYB2 detected by co-immunoprecipitation assays. The 5′Δ5N-Pac and pPTOP3β stable transfectants were cultured in encystation medium for 24h. Proteins from cell lysates were immunoprecipitated using anti-HA antibody conjugated to beads. The precipitates were analyzed by Western blotting with anti-HA, anti-TOP3β, anti-MYB2, and anti-ISCS antibodies, respectively, as indicated. (E) TOP3β and MYB2 interaction confirmed by Far Western blot analysis. Recombinant MYB2-N (residues 1–410) and ISCS proteins with a V5
tag at its C terminus was purified by affinity chromatography and detected by anti-V5 antibody in Western blot analysis (bottom panels). Far Western blot analysis was performed using the purified recombinant MYB2-N. The ISCS protein was used as a negative control. MYB2-N and ISCS were subjected to separation by SDS-PAGE, transferred onto a membrane, refolded in renaturation buffers, and incubated with lysate from the pPTOP3β stable transfectants as in Fig. 7D. Bound TOP3β-HA was detected with immunoblot using anti-HA antibody (upper panels). The purified recombinant MYB2-N and ISCS proteins on the membrane were detected using anti-V5 antibody (lower panels). TOP3β-HA bound to MYB2-N but not to ISCS.

Fig. 8. Decrease in expression of cwp1-3 and myb2 by targeted disruption of the top3β gene during vegetative growth. (A) Diagrams of the pgCas9 and pTOP3βtd plasmids. In construct pgCas9, the cas9 gene is under the control of gdh promoter (striated box) and 3’ untranslated region of the ran gene (dotted box) and its product has a C-terminal nuclear localization signal (filled gray box) and an HA tag (filled black box). In construct pTOP3βtd, a single gRNA is driven by the Giardia U6 promoter. The single gRNA includes a guide sequence targeting 20-nucleotide of the top3β gene (nt 135–154), which is located upstream of three nucleotides of protospacer-adjacent motif (NGG sequence). pTOP3βtd also has the HR template cassette which contains the 5’ and 3’ flanking region of the top3β gene as homologous arms and the pac selectable marker. The Cas9/gRNA cutting site in the genomic top3β gene is indicated by a red arrow. After introducing a double-stranded DNA break in the top3β gene, replacement of the genomic top3β gene with the pac gene will occur by HR. The pgCas9 and pTOP3βtd constructs were transfected into G. lamblia WB trophozoites. An NHEJ inhibitor, SCR7, was added to
increase HR. The TOP3βtd stable transfectants were established under puromycin selection. The control cell line is trophozoites transfected with double amounts of 5'Δ5N-Pac plasmid (Fig. 1D) and selected with puromycin. PCR1/2 were used for identification of clones with targeted disruption. (B) Partial replacement of the top3β gene with the pac gene in the TOP3βtd cell line confirmed by PCR. Puromycin was kept in the TOP3βtd and control cell lines. Genomic DNA was isolated from the TOP3βtd and control cell lines cultured in growth medium (vegetative growth, Veg). PCR was performed using primers specific for top3β (PCR1 in panel A), pac (PCR2 in panel A), cwp1, cwp2, and ran genes, respectively. Products from the cwp1, cwp2, and ran genes are internal controls. (C) Partial disruption of the top3β gene in the TOP3βtd cell line confirmed by real-time PCR. Real-time PCR was performed using primers specific for top3β, cwp1, cwp2, and ran genes, respectively. The top3β, cwp1, and cwp2 DNA levels were normalized to the ran DNA level. –Fold changes in DNA levels are shown as the ratio of DNA levels in the TOP3βtd cell line relative to the control cell line. Results are expressed as the means ± 95% confidence intervals S.E. (error bars) of at least three separate experiments. *p<0.05 was considered significant and the value was shown. (D) Targeted disruption of the top3β gene increased G418 sensitivity. The TOP3βtd and control cell lines were subcultured at an initial density of 1×10^6 cells/ml in growth medium containing 518μM G418 for 24h and then subjected to cell count. An equal volume of ddH2O was added to cultures as a negative control. The sum of total cells is expressed as a relative expression level over control. Values are shown as means ± 95% confidence intervals S.E. of three independent experiments. *p<0.05 was considered significant and the value shown. The viability of the TOP3βtd cell line decreased compared to the control cell line. (E) Cyst formation decreased by targeted disruption of the
top3β gene in the TOP3βtd cell line during vegetative growth. The control and TOP3βtd cell lines were cultured in growth medium and then subjected to cyst count as described under “Materials and Methods” and Fig. 3D. (F) Targeted disruption of the top3β gene decreased the CWP1 level in the TOP3βtd cell line during vegetative growth. The control and TOP3βtd cell lines were cultured in growth medium and then subjected to SDS-PAGE and Western blot analysis as described in Fig. 3A. The blot was probed with anti-TOP3β, anti-CWP1 and anti-RAN antibodies, respectively. The intensity of bands from three Western blot assays was quantified as described in Fig. 3A. (G) Decrease in expression of cwp1-3 and myb2 by targeted disruption of the top3β gene in the TOP3βtd cell line during vegetative growth. The control and TOP3βtd cell lines were cultured in growth medium and then subjected to quantitative real-time RT-PCR analysis using primers specific for top3β, cwp1, cwp2, cwp3, myb2, ran, and 18S ribosomal RNA genes, respectively, as described in Fig. 1B.

Fig. 9. Increase of encystation-induced cwp1-3 genes during differentiation into cysts. The genes encoding key components of the cyst wall, cwp1-3, are up-regulated by TOP3β, MYB2, and other transcription factors during differentiation into cysts. These factors can bind to cis-acting elements, such as box1-4 or AT-rich initiator (Inr) of the cwp1-3 promoter to activate cwp1-3 transcription. TOP3β, MYB2, and other transcription factors, can form complexes and recruit RNA polymerase II to activate cwp1-3 transcription. CWP1 was present in vegetative trophozoite stage at a lower level. During encystation, more CWP1 is produced by these factors. During encystation, the increase of MYB2, TOP3β, and other transcription factors may further induce CWP1 expression, resulting in more cyst formation.
