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Topoisomerase 3b is dispensable for replication of a positive-sense RNA virus--murine coronavirus

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A recent study demonstrated that a DNA-RNA dual-activity topoisomerase complex, TOP3B-TDRD3, is required for normal replication of positive-sense RNA viruses, including several human flaviviruses and coronaviruses; and the authors proposed that TOP3B is a target of antiviral drugs. Here we examined this hypothesis by investigating whether inactivation of Top3b can inhibit the replication of a mouse coronavirus, MHV, using cell lines and mice that are inactivated of Top3b or Tdrd3. We found that Top3b-KO or Tdrd3-KO cell lines generated by different CRISPR-CAS9 guide RNAs have variable effects on MHV replication. In addition, we did not find significant changes of MHV replication in brains or lungs in Top3b-KO mice. Moreover, immunostaining showed that Top3b proteins are not co-localized with MHV replication complexes but rather, localized in stress granules in the MHV-infected cells. Our results suggest that Top3b does not have a universal role in promoting replication of positive-sense RNA virus, and cautions should be taken when targeting it to develop anti-viral drugs.

Topoisomerases are essential to solve topological problems produced during essential cellular processes on DNA, including replication, transcription, recombination, and chromosome segregation; and are targets for multiple successful anti-cancer drugs (Pommier et al. 2022). Our group and others have shown that a type IA topoisomerase conserved from human to Drosophila, TOP3B, is a dual-activity topoisomerase that can change topology for not only DNA, but also RNA (Xu et al. 2013; Siaw et al. 2016). TOP3B is the only topoisomerase containing a conserved RNA-binding domain, which is critical for its binding to mRNA and for its RNA (and DNA) topoisomerase activity. Top3b forms a stoichiometric complex with a conserved tudor-domain-containing protein, Tdrd3 (Stoll et al. 2013; Xu et al. 2013; Yang et al. 2014). Current evidence shows that TOP3B-TDRD3 directly participates in transcription on DNA and translation on mRNAs (Joo et al. 2020; Su et al. 2022). TOP3B-TDRD3 can directly bind and regulate translation and turnover of a subset of mRNAs through topoisomerase activity-dependent or independent mechanisms (Su et al. 2022). These data support our hypothesis that mRNA metabolism may produce topological problems that depend on an RNA topoisomerase, TOP3B, to solve (Xu et al. 2013; Ahmad et al. 2017).

Interestingly, a recent paper by Prasanth and colleagues showed that RNAi depletion or knockout (KO) of either TOP3B or TDRD3 in cultured human cell lines can inhibit replication of several positive-sense RNA viruses, including Dengue, Zika, SARS-CoV-1 and SARS-CoV-2 (Prasanth et al., 2020). The authors thus proposed that TOP3B is an attractive anti-viral target. This study can lead to two important directions for future research. First, because long double-stranded RNAs are produced during the replication of positive-sense RNA viruses, it is possible that Top3b can relieve the topological stress during the viral RNA replication. If this is the case, viral RNA replication will be a valuable model to identify the RNA topological stress that is solved by TOP3B. Second, considering that the world is still troubled by the COVID-19 pandemics caused by a human coronavirus, SARS-CoV-2, it will be important to carefully examine the proposal whether TOP3B is an anti-viral target. If it is, Top3b inhibitors should be developed to mitigate pandemics caused by SARS-CoV-2, and possibly other pathogenic positive-sense viruses.

Here we examined this proposal by investigating whether inactivation of TOP3B or TDRD3 can inhibit replication of a positive-sense RNA virus, murine coronavirus (Mouse Hepatitis Virus (MHV)) by using not only RNAi depletion and KO in cell lines, but also a KO mouse model.

Keywords:
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ABSTRACT

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Here we examined this proposal by investigating whether inactivation of TOP3B or TDRD3 can inhibit replication of a positive-sense RNA virus, murine coronavirus (Mouse Hepatitis Virus (MHV)) by using not only RNAi depletion and KO in cell lines, but also a KO mouse model.
MHV belongs to the same β-coronavirus sub-family as does human SARS-CoV-2, and the two viruses share similar structures and life cycles. The FDA-approval anti-SARS-CoV-2 drug, Remdesivir, can inhibit MHV replication with high efficiency, which supports a conserved mechanism of replication between the two viruses (Agostini et al. 2018). One advantage of MHV is that it can be safely handled in regular BSL2 lab settings, so that it is a popular model for mechanistic studies and drug discoveries for coronavirus (Weiss and Leibowitz 2011).

To study the functions of Top3b in MHV replication, we inactivated this gene by CRISPR-Cas9 in one of the MHV mouse host cell lines, 17Cl-1 (Sturman and Takemoto 1972), by using two different sgRNAs: one targets the N-terminal region of Top3b (KO1), whereas the other targets the catalytic core domain containing the residue (Y336) essential for topoisomerase reaction (KO2) (Fig. 1A). Immunoblotting revealed that Top3b protein levels were undetectable in whole cell lysates from these two KO cell lines (Fig. 1B), indicating successful inactivation of the

**Fig. 1.** Top3b-KO and Tdrd3-KO cells generated from different sgRNAs did not consistently suppress MHV replication. A) Scheme of Top3b protein with the locations of the designed sgRNAs of CRISPR-Cas9. Y336 is the key amino acid for Top3b catalytic activities. Red and blue arrows next to each sgRNA indicate MHV replication is increased and decreased in Top3b-KO cells generated by that sgRNA respectively. The increased or decreased levels are represented by the arrow numbers. B) Top3b protein levels in WT, Top3b-KO1 and Top3b-KO2 17Cl-1 cell lines were detected by Western blot. C) Relative levels of MHV mRNA detected by RT-qPCR from WT, Top3b-KO1 and Top3b-KO2 17Cl-1 cell lines infected by MHV. The cells were infected with 0.5 MOI MHV and harvested for RNA extraction at 8 h post-infection (hpi). D) Firefly luciferase activities from WT, Top3b-KO1 and Top3b-KO2 cells infected by MHV with nsp2-luciferase reporter (MHV-luci). The cells were infected with 0.5 MOI MHV-luci and harvested for luciferase assay at 5 hpi. WT cells without MHV infection were used as negative control (NC). E) Green fluorescence signals of WT, Top3b-KO1 and Top3b-KO2 cells infected by 0.02 MOI MHV with an EGFP reporter in a 96-well plate. The images were captured at 24 hpi. F) The virus titers of the samples from a 96-well plate with WT, Top3b-KO1, and Top3b-KO2 cells infected by 0.02 MOI MHV with an EGFP reporter in a 96-well plate. The images were captured at 24 hpi. G) Relative firefly luciferase activities of WT and Top3b-KO cells infected by MHV-luci. Top3b-KOs were generated by sgRNA 2, 3, 4 and 5. The cells were infected with 0.5 MOI MHV-luci and harvested for luciferase assay at 5 hpi. H) Tdrd3 and Top3b protein levels in WT, Tdrd3-KO1 and Tdrd3-KO2 17Cl-1 cell lines were detected by Western blot. Gapdh was used as a loading control. I) Relative fly luciferase activities of WT, Tdrd3-KO1 and Tdrd3-KO2 cell lines infected by MHV-luci. The cells were infected with 0.5 MOI MHV and harvested for luciferase assay at 8 hpi.
protein. The sequencing results of cloned PCR products amplified from the regions around the CRISPR-edited sites of Top3b-KO1 and -KO2 cells showed that the edited alleles all have frame-shift deletions, leading to out-of-frame stop codons and thus loss of large portion of Top3b protein (Fig. S1A). Because these out-of-frame stop codons occurred in exons that have introns downstream (not the last exon), the mutant mRNAs will be subjected to non-sense mediated decay (NMD), so that they are not expected to produce any truncated proteins. Indeed, immunoblotting failed to detect the presence of any truncated Top3b proteins (Figs. S2A–B).

We then monitored MHV replication after infection in WT and Top3b-KO cells by RT-qPCR. Comparing to the WT control, MHV mRNA levels were decreased about 4-fold in Top3b-KO1 cells, but strongly increased (about 20-fold) in Top3b-KO2 cells (Fig. 1C). We further used MHV strains with luciferase (MHV-luci) and EGFP (MHV-EGFP) reporters to detect MHV replication (Das Sarma et al., 2002; Freeman et al. 2014). Similar to the changes of MHV mRNA levels, the luciferase activities from MHV-luci were decreased in Top3b-KO1 but strongly increased in Top3b-KO2 cells (Fig. 1D). The GFP signals from Top3b-KO1 and Top3b-KO2 cells infected by MHV-EGFP were also significantly decreased and increased, respectively (Fig. 1E). We used plaque assay to confirm that the viral titers were decreased in Top3b-KO1 but increased in Top3b-KO2 cells infected by MHV-EGFP (Fig. 1F). These results suggest that MHV replication is suppressed in Top3b-KO1 cells whereas enhanced in Top3b-KO2 cells.

To examine whether the opposite results from Top3b-KO1 and -KO2 may be due to off-target effects, we generated three more Top3b-KO cell lines, Top3b-KO3, -KO4 and -KO5, by different guide RNAs (Fig. 1A). Top3b-KO3 was generated by a guide RNA targeting the core catalytic domain of Top3b (similar to KO2); whereas KO4 and KO5 were generated by guide RNAs targeting N-terminal region of Top3b (similar to KO1). The individual Top3b-KO clones were selected based on screening of Top3b protein levels by Western blotting (Fig. S3) The edited effects in some clones were also confirmed by PCR (Fig. S1B). The obtained Top3b-KO clones were then infected with MHV-luci, and their luciferase activities were detected and compared to that of WT cells. We found that MHV-luci levels in 3 individual clones of Top3b-KO3 were strongly increased (>20-fold), which resemble the results obtained in Top3b-KO2 cells (Fig. 1G). In contrast, MHV-luci levels in 4 individual clones of Top3b-KO4 and 3 individual clones KO5 only showed either modest decreases or increases (<5-fold), or no obvious changes (<2-fold) (Fig. 1G). The results from Top3b-KO4 and -KO5 are somewhat similar to those from the single clone of Top3b-KO1, which showed moderate reduction MHV replication (Fig. 1C). Overall, our results revealed that Top3b-KO by different sgRNAs have variable effects on MHV replication. One possible explanation for the observed variable effects may be that some KO clones have lost of Top3b protein, and thus have loss-of-function effects, whereas other clones may have produced truncated Top3b proteins, so that can have gain-of-function effects. We used two Top3b antibodies to test this possibility. However, both Top3b monclonal and polyclonal antibodies did not detect truncated forms of Top3b in Top3b-KO cell lines (Fig. S2A-B). We need to note that the Western blot by Top3b polyclonal antibody had many nonspecific bands, and a better polyclonal antibody is needed to examine this issue in future. Another possibility is that the variable effects could be due to the off-target effects of sgRNAs.

We next studied whether TDRD3-KO cells generated by different sgRNAs can have similar effects on MHV replication. TDRD3 is a key interacting partner of Top3b, and can increase the catalytic activity (Siaw et al., 2016) and protein stability of Top3B (Stoll et al., 2013; Xu et al., 2013). TDRD3-KO has also been reported to suppress replication of several positive-sense RNA viruses (Prasanth et al., 2020). We generated Tdrd3-KO 17CI-1 cell lines with two different sgRNAs. Immunoblotting confirmed the absence of Tdrd3 protein in each cell line (Fig. 1H). The edited site in Tdrd3-KO2 line was confirmed by genomic PCR and subsequent sequencing, which revealed a frame-shift deletion, similarly to edited sites observed in Top3b-KO cells (Fig. S2A). Top3b protein levels were drastically decreased in both Tdrd3-KO cells (Fig. 1H), which is consistent with previous results that TDRD3 stabilizes TOP3B (Stoll et al., 2013; Xu et al., 2013). MHV-luci levels were found to be modestly decreased in one clone (<3-fold), but largely unchanged in another (<1.5-fold) (Fig. 1I). These results are similar to those of Top3b-KO cells, and suggest that inactivation of the entire TOP3B-TDRD3 complex by different sgRNAs have variable effects on MHV replication. Below, we used two additional approaches to study the effect of loss of Top3b on MHV replication.

We first examined the effects of Top3b depletion on MHV replication, using three different siRNA oligos. All three siRNAs had similar effects in decreasing Top3b mRNA levels (about 80%) (Fig. S4A). MHV levels remained largely unchanged in cells treated by two of the three siRNAs (fold change<20%), and was reduced by 5-fold in cells treated by the third siRNA oligo (Fig. S4B). The results from the first 2 siRNA oligos, but not the third oligo, are consistent with data from a Top3b-KO mouse model (see below), suggesting that Top3b is dispensable for MHV replication. The negative effect of the third siRNA oligo is likely due to the off-target effect of this particular oligo. Overall, data from our Top3b-KO and siRNA depletions studies are largely inconsistent with the previous findings in human cell line that TOP3B is required for efficient replication of coronavirus replication (Prasanth et al., 2020).

We then tested whether MHV replication was affected in a Top3b-KO mouse line, which should be a loss of function mutant because the conserved topoisomerase catalytic core domain is disrupted by homologous recombination (HR)-mediated gene targeting (Kwan and Wang 2001). One advantage of HR-based gene targeting is that it has much higher specificity, because its homology regions are several thousand base pairs, about a hundred-fold longer than those in sgRNAs and siRNAs (about 20 bps). Thus, it should largely avoid the off-target effects of the latter two strategies, and the data should be more convincing. We used two common MHV administration methods to infect the mice and analyzed the virus levels in the infected tissues: intracranial injection to infect the brains (Das Sarma et al., 2002), and intranasal inoculation to infect the lungs (Koo et al. 1999). We harvested the tissues at day 3 and 5 post MHV infection, because previous studies have shown that MHV levels are relatively high at these time-points (Das Sarma et al., 2002; De Albuquerque et al. 2006). The day-3 data point should largely reflect MHV replication, whereas the day-5 point can reflect a combined effect of viral replication and clearance. We found that at day-3 after intracranial infection, the MHV levels in the brains did not show significant differences between WT and Top3b-KO mice, by RT-qPCR (Fig. 2A) and plaque assays (Fig. 2B), indicating that Top3b inactivation had no significant effects on MHV replication. Similarly, at day-5 after intranasal inoculation, the MHV mRNA levels in lungs or livers did not show significant differences between WT and Top3b-KO mice either (Fig. 2C), suggesting that Top3b inactivation may not significantly alter replication and/or clearance of MHV.

A potential function of Top3b in MHV replication is to resolve the topological problems during viral RNA replication, because this process can produce long double-stranded (ds) RNA, which may resemble the long dsDNA in generating topological stress. A multiprotein complex containing RNA-dependent RNA polymerase binds and replicates MHV genomic RNA. Nsp2 is a component of this replication complex, and Nsp2-GFP has been used as a marker to determine the localization of the complex (Freeman et al., 2014). We first confirmed that Nsp2-GFP was co-localized with double-stranded (ds) RNA detected by J2 antibody in MHV-infected 17CI-1 cells (Fig. 3A). Then, we performed a double staining experiment, and observed no obvious co-localization between signals of Top3b and Nsp2-GFP (Fig. 3B). The Top3b immunostaining was specific since the signals were undetectable in Top3b-KO cells infected by MHV-GFP-Nsp2 (Fig. S5A). The lack of co-localization between Top3b and viral replication complex is consistent with the functional data above that Top3b is largely dispensable for MHV replication.

We found that Top3b is accumulated in granule-like dots in MHV-
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infected 17C1-1 cells (Fig. 3B). Top3b is known to localize to RNA stress granules in cells under oxidative stress, and MHV infection can induce stress granule formation (Raaben et al. 2007; Xu et al. 2013; Youn et al. 2018). This prompted us to investigate whether the granule-like dots are virus-induced RNA stress granules. Indeed, we found that most of these Top3b-containing granules co-localize with G3bp1, a stress granule marker, in MHV infected cells (Fig. 3C, quantification in Fig. S5B). These results show that Top3b is localized in stress granules instead of MHV replication complexes. However, it should be cautioned that although we did not observe colocalization of Top3b and MHV replication complexes, our data do not exclude the possibility that they have dynamic interactions that cannot be detected by our current method.

In conclusion, our results from Top3b/Tdrd3-KO and -depletion cell lines, and Top3b-KO mice are inconsistent with the conclusion by Prasanth and colleagues that TOP3B is required for efficient replication of positive-sense RNA viruses. It should be cautious that our conclusion is based on one coronavirus (MHV) in one host (mouse). Nevertheless, a variety of genome-wide screens have been performed to identify host factors required for human coronavirus (SARS-CoV-2) replication in human host, and these screens fail to identify either TOP3B or TDRD3 as
a positive hit (Baggen et al. 2021; Schneider et al. 2021; Wang et al. 2021; Wei et al. 2021). These negative data are consistent with our conclusion that TOP3B-TDRD3 is dispensable for replication of coronavirus.

We should point out that although our data are inconsistent with those by Prasanth and colleagues regarding the roles of TOP3B-TDRD3 in coronavirus replication, we still propose that it is worth investigating whether TOP3B could be an anti-viral target. Our rationale is that topoisomerase-based anti-cancer drugs are not simply inhibitors of enzymes, but are also “poisons” with a critical gain-of-function—they trap the topoisomerase-DNA cleavage complexes (the intermediates of topoisomerase reactions), and prevent the broken DNA from realignment (Fig. S6 left). This creates damaged DNA containing a protein-DNA crosslink and a DNA break, which can block DNA replication (Delgado et al. 2018; Thomas and Pommier 2019). Similarly, if compounds that target TOP3B can trap its topoisomerase-RNA cleavage complex on viral RNAs, the resulting damaged RNA could inhibit viral RNA replication (Fig. S6 right). A TOP3B mutant protein has been reported to form cleavage complexes on cellular RNAs (Saha et al. 2020). We propose that as long as TOP3B can also form cleavage complexes on viral RNAs, it could be a target for anti-viral drug.

Note added in-proof

We have communicated our findings that TOP3B-TDRD3 is dispensable for MHV replication to Dr. Garcia-Blanco and colleagues. Dr. Garcia-Blanco’s group have since repeated the SARS-CoV-2 infection experiments with a reporter SARS-CoV-2 virus in human TOP3B-KO and TDRD3-KO cells, and found that the viral replication is not significantly reduced in TOP3B-KO or TDRD3-KO cells (Dr. Garcia-Blanco, personal communication). The effect on Zika virus replication was still noted, however. His group is currently characterizing all KO cell lines to determine the basis for the different results with SARS-CoV-2. It remains unclear why their current and previous data are different. Nevertheless, the current data from both his and our groups suggest that TOP3B-KO cell lines generated by different sgRNAs may result in variable phenotypes, and the conclusion that TOP3B is required for efficient coronavirus replication requires reconsideration.

Materials and methods

See Supplemental Information.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced to the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2022.105451.

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