Skoltech
Skolkovo Institute of Science and Technology

Thesis Changes Log

Name of Candidate: Sutormin Dmitry
PhD Program: Life Sciences
Title of Thesis: Regulation of bacterial genome topology by topoisomerases
Supervisor: Prof. Konstantin Severinov

The thesis document includes the following changes in answer to the external review process.

Prof. Mikhail Gelfand did not have any comments.

Comments from **Prof. Konstantin Lukyanov**:

1. Pages 37, 50: Some words are in red, probably left over from a draft.
   Response:
   The text color was corrected.
2. Fig. 26: Designations on the panel D are invisible (too small). Also, designations on the panel F are quite small.
   Response:
   Designations for panels D and F were substantially enlarged.

Comments from **Prof. Marc Drolet**:

1. Page 23: topo i also is enriched in Mycobacterium
   Response:
   The sentence was changed to include additional information about the TopoI enrichment and the corresponding paper was cited. “ChIP-Seq experiments demonstrated that mycobacterial gyrase (in *M. tuberculosis*) and Topoisomerase I (in *M. smegmatis*) are significantly enriched at the chromosomal replication terminus region, which suggests that they substitute TopoIV [39,63,108,109].”
2. Page 31: There areno evidence of topo iii acting with RecQ in E. coli. See recent papers of Marians and also Drolet. In fact this does not seem to happen. Ok below!!!!!
   Response:
   The following sentence was added to the Thesis. “Should be noted, that suggested cooperation is lacking strong evidence *in vivo* and was disputed by genetic experiments [188–191].”
3. Page 70: ??? not related to twin-model
   Response:
   The sentence was corrected as follows. “Average distance between leading replisomes in early replicating cells and emerging replication-induced gyrase enrichment was -15 ± 30 kb (± standard deviation) (Figure 24E), indicating colocalization, which is in accordance with gyrase attraction by positive supercoiling generated by moving DNA polymerase.”
4. Page 80: What will be the role if any of topo I in transcription termination?

Response:
The sentence was added as follows. “The roles of associated EcTopoI in transcription termination process, if any, could be in facilitation of DNA duplex restoration after the RNAP complex dissociation.”

5. Page 88: text highlighted
   Response:
   The sentence was corrected as follows. “Intriguingly, compared to regions upstream of TUs, cleavage activity of EcTopoI was significantly lower…”
6. Page 90: or activity of topo i need to be attenuated in gene bodies because negative supercoiling upstream may facilitate transcription by the incoming RNAP????
   Response:
   The sentence was corrected as follows: “Deacetylation may be triggered by conformational changes within the RNAP:EcTopoI complex caused by RNAP stalling or by extensive torsional stress (e.g., excessive negative supercoiling).”
7. Page 90: Explain better please
   Response:
   Sentence was added: “If true, RNAP:EcTopoI (G116S M320V) complexes, stalled due to the catalytic activity of the “intrinsically poisoned” EcTopoI double mutant, can be recognized by Mfd or NusA-UvrD and be removed by nucleotide-excision repair pathway.”
8. Page 91: For fast growing bacteria
   Response:
   It is hard to tell whether growth rate can define the “open” or “closed” types of a twin-domain model. In slow growing bacteria, like *Mycobacterium*, key enzymes have proportionally lower activities establishing a new equilibrium in the formation and relaxation of supercoils. The following text was added to page 92. “In contrast, for *Mycobacterium* interactions between enzymes are likely playing a critical role in the establishing of the “closed” model as M RNAP elongation rate and M DNA gyrase turnover rate are proportionally slower than for *E. coli* enzymes (approximately 10 times slower) [53,108,279]. Of note, MtTopoI relaxation activity is also lower than EcTopoI activity, but to less extent [270]”.

Comments from **Prof. Anthony Maxwell**:

1. The Chapter 4 could do with more preamble to give context.
   Response:
   A short preamble was added to page 49. “To understand the role of topoisomerases in regulation of genome topology and, particularly, in dissipation of DNA supercoiling, a method for detection of topoisomerase activity is needed. In this section I introduce such a method, named Topo-Seq, and describe findings obtained with it for key bacterial topoisomerases - DNA gyrase, TopoIV, and TopoI. With Topo-Seq, the activity of topoisomerases was linked to transcription and replication genome-wide and topoisomerase DNA motifs were characterized.**”**
2. Fig. 9 could do with a key to symbols.
   Response:
   Figure legend was extended.
3. P. 53: explain the effects of increased drug concentration.
   Response:
   A sentence was added. “Observed proportionality indicates that increased drug concentration induces more stable cleavage complexes, which are detected by sequencing.”
4. P. 57: Fig 15 – is the increase in GCSs for high versus low transcribed genes really significant? (oxo result).
   Response:

The following passage was added. “Whole-genome analysis revealed that in general less GCSs associate with poorly transcribed transcription units (TUs) than with highly transcribed ones: 223 vs 552 GCSs for Cfx (t-test, p-value<2.5e-7), 35 vs 88 for Micro (t-test, p-value<1.6e-4), 339 vs 498 for Oxo (t-test, p-value<3.9e-3). The same conclusions were made with gene-centric analysis for poorly expressed and highly expressed genes: 569 vs 1128 GCSs for Cfx (t-test, p-value<4.9e-9), 88 vs 183 for Micro (t-test, p-value<3.0e-5), 815 vs 1113 for Oxo (t-test, p-value<1.7e-4) (Figure 15 A-C). Association and depletion of cleavage sites for highly and poorly transcribed TUs, respectively, is in line with observations made for M. tuberculosis gyrase [63].”

5. P. 59: is there much difference +/- rif? (Fig 16).
   Response:
   As indicated in the text, “Rifampicin did not affect either overall shape of gyrase motif, or local cleavage properties characteristic of Cfx (Figure 16B-C)”
6. P. 62: what is the significance of the BIME results?
   Response:
   We demonstrated that BIME sequences are recognized and cleaved by gyrase when transcription is inhibited, and these sequences have higher than average score indicating their similarity with gyrase motif. The following passage was added: “indicating that BIME cleavage by gyrase is transcription independent. “
7. P. 67: role of Azamat?
   Response:
   Text was added to Paragraph’s 4.12 preamble. “Azamat assisted with samples preparation during the time-course experiment.”
8. P. 70: why HCl and NaOH?
   Response:
   The following passage was added. “To increase solubility, Cfx stock solution was prepared in 0.1 mM HCl (30 mM Cfx, pH~4), and oxolinic acid stock solution was prepared in 5 mM NaOH (3 mM Oxo, pH~11.5). To control for toxicity of high and low pH, MICs of HCl and NaOH were also determined: 1.5 mM and >5 mM for HCl and NaOH, respectively. Cfx had a much lower MIC compared to Oxo (5.9 μM vs 95 μM, respectively) and was used in further Topo-Seq experiments in a concentration 1.5 mM (~256*MIC). Concentration of the acid in the 1.5 mM Cfx working solution is significantly lower than HCl MIC (5 μM vs 1.5 mM) and, therefore, likely does not introduce bias.”
9. P. 87, Fig. 33D – grey rectangles are not clear.
   Response:
   Explanation is given in the figure legend. “Amplified regions used for ChIP-qPCR and affinity measurements are indicated with grey rectangles.”
10. P. 87, Fig. 33E – why use EMSA?
    Response:
    EMSA was used for quick and cheap estimation of affinity. For in depth investigation of EcTopoI binding preferences EMSA in combination with MST was used later (see Fig. 4h-I in Sutormin et al., 2022, NatCom. These data were not included in Thesis).
11. P. 90 – discuss the annihilation of +ve/-ve supercoils by RNA polymerase.
    Response:
    The sentence was extended as follows. “I speculate that in HETUs, where EcTopoI remains inactive, RNAP molecules move in convoy and mutually annihilate positive and negative supercoils generated by polymerases elongating consequently [274,275]”
12. The paragraphing is not consistent.
    Response:
    Paragraphing was fixed and made consistent.
13. There is some text in red that needs to be altered.
    Response:

The text color was corrected.

Comments from **Dr. Keir Neuman**:

1. P. 3: attracted gives the sense of being drawn to these regions. I think that "associated with regions of transcription and replication" is more appropriate.
   Response:
   The sentence was changed correspondingly.
2. P. 12: I think that it would be worth mentioning that plectonemes arise in unconstrained writhe whereas solenoids are only associated with writhe constrained by bound proteins.
   Response:
   Information of constrained and unconstrained types of writhe was added.
3. P. 13. The number is not arbitrary - there is a probabilistic distribution of linking number changes, but the number is not arbitrary.
   Response:
   Term “arbitrary” was removed.
4. P. 13: I do not think that the number of ATP hydrolyzed per cycle has been firmly established. there is evidence for at least one being required but no direct evidence for two ATP molecules being hydrolyzed. It is likely that two molecules are hydrolyzed, but not firmly established.
   Response:
   The sentence was changed as follows. “hydrolyzing at least one ATP molecule in the process (hydrolysis of two molecules is proposed, but has not been directly shown yet)”
5. P. 14: I think that binding is separate from bending, but bending is required for cleavage.
   Response:
   The sentence was changed. “N-gate closure further enhances bending of G-segment, which together with hydrolysis of the first ATP molecule to ADP triggers cleavage of the G-segment by catalytic site tyrosyl residues of the WHDs…”
6. P. 15: My impression from speaking with James Berger recently is that the C-gate opening is triggered by the religations of the DNA at the DNA gate and the associated disassociation of the tyrisines from the DNA and the relaxation of the WHD, which is coupled to C-gate opening. this order of events also provides greater DNA stability since the c-gate will only open after the DNA gate is sealed.
   Response:
   The paragraph was revised.
7. P. 18. The discussion of poisoning of type II topoisomerase poisons is focused on their role in disrupting metal ion interactions. This should be expanded to include the notion of “interfacial inhibitors” that is an important aspect of inhibition.
   Response:
   The paragraph was expanded. “Described drugs belongs to a wider group of ‘interfacial inhibitors’, which target macromolecular interfaces and typically stabilize normally transient intermediates [51,52]. According to this point of view, quinolone and fluoroquinolone drugs sterically prevent ligation of DNA break by intercalating between DNA nucleotides to be sealed with a phosphodiester bond.”
8. Page 29. I would like to see a more detailed discussion of r-loop formation starting with a negatively supercoiled DNA molecule and including the impact of R-loop formation in capturing negative supercoiling (-Lk) on the overall process.
   Response:
   A paragraph was extended considerably. “Hypernegative supercoiling (a level of negative supercoiling which is much higher than in wild type cells) is a hallmark of *topA* mutants resulting from uncompensated gyrase activity [160]. It was observed that hypernegative supercoiling is interconnected with formation and stabilization of R-loops - RNA-DNA heteroduplexes formed

when nascent transcripts anneal to the template DNA strand upstream of the transcribing RNA polymerase (RNAP) [160–162]. First, R-loops have been recently detected in *topA* mutants (exhibiting hypernegative supercoiling) by dot-blots assays with an RNA:DNA hybrid-specific antibody [163]. Second, R-loops formation during *in vitro* transcription was found to be dependent on negative supercoiling of a template plasmid and it was increased for hypernegatively supercoiled substrate [160,162]. Reciprocally, formation of a hypernegatively supercoiled plasmid form was reproduced during *in vitro* transcription in the presence of gyrase and it was dependent on R-loops formation, as treatment with RNase A or RNase HI (specifically removes R-loops) had a suppressive effect [160,161,164]. To explain the interdependence of hyernegatively supercoiling and R-loops formation in cells lacking topoisomerase I, an autocatalytic model was proposed. According to the model, elongating RNAP triggers this process, as it introduces negative supercoiling upstream and positive supercoiling downstream (a substrate for gyrase) and produces nascent RNA which can form an R-loop. Gyrase converts positive supercoiling into negative, and this promotes nucleation of an R-loop. Since one DNA strand is unpaired in the R-loop, a local hub accumulating excessive negative supercoiling is created, masking negative supercoiling and leaving the nearby DNA relaxed, which was observed *in vitro* using transcription assay [162]. Same time, RNAP continues elongation and produces more negative and positive supercoiling, the latter of which is relaxed by gyrase. Excessive negative supercoiling leads to R-loops propagation and absorption of more negative supercoiling, therefore preparing a substrate for gyrase together with RNAP. A positive feedback loop is created, leading to further accumulation of R-loops and additional negative supercoiling (negative supercoiling → R-loops propagation → absorbtion of negative supercoiling → increase gyrase activity → excessive negative supercoiling) (**Figure 8**) [165]. Actually, in the presence of nascent transcripts as a source of RNA component of R-loops, formation of hypernegatively supercoiled plasmid requires only gyrase activity and can be observed without active transcription [164]. Should be noted that the outlined model and connected body of data indicate that in normal cells R-loops may play a role in supercoiling management as efficient supercoiling accumulators [166]."

9. P. 30: Keep formatting the same - maintain numbered references
   Response:
   Reference style was corrected.
10. P. 38: This is confusing - maybe a table would be helpful since it seems that there are 4 or 6 different conditions - or did you add both 0.9 and 10 u cipro?
    Response:
    A table was added.
11. P. 55: This should be in some inverse units other than Hz - this should be in inverse bp or or some other inverse unit. likewise the graph in part D should be in inverse nt rather that Hz. this corresponds to a spatial (or nucleotide) frequency rather than a temporal frequency.
    Response:
    Figure and the figure legend were corrected.
12. P. 61: I think that gyrase has a higher affinty for postively supercoiled DNA or something along these lines rather than "being attraced to" positively supercoiled DNA, which gives it an action that it does not posses.
    Response:
    The sentence was corrected. "…suggesting that transcription is a strong factor that determines gyrase enrichment in downstream DNA."
13. P. 65: several comments on gyrase association with positively supercoiled DNA and active transcription.
    Response:
    The sentence was changed as followed. "…supporting the idea that gyrase has higher affinity to positively supercoiled DNA and is associated with active transcription (**Figure 21E**)."

14. P. 69: be associated with

    Response:

    The sentence was changed. “I propose that replication initiation and/or progression of replisomes close to the origin region may be associated with increased gyrase activity likely via increased positive supercoiling generated.”

15. Page 72. I would like to see more discussion on possible explanations as to why there is more cleavage by topo IV in the Gyrase S83l background. The notion of more Cpx molecules seems unlikely to me.

    Response:

    A passage was added providing an alternative hypothesis. “Alternatively, GyrA S83L mutant could have a decreased DNA binding and/or supercoiling activity, which might lead to proportionally increased TopoIV activity detected with Topo-Seq. The latter hypothesis is supported by the observation that in *gyrA S83L* background DNA is less negatively supercoiled implying the reduced activity of the mutated protein [212]. However, other groups reported that supercoiling is not affected by this mutation and that this mutation has a neutral effect on cell fitness [252,253]. Activity of GyrA S83L-containing gyrase should be directly compared to the wild-type enzyme *in vitro* and amounts of gyrase, TopoIV, and TopoI should be compared between *wt* and mutated strains to test this hypothesis.”

16. Page 75. The in vitro measurements of the sequence specificity of topo IV should be referenced and discussed in relation to the findings presented in the thesis.

    Response:

    To address this commentary, a passage was added, and a new figure (Figure 26G) was created. “Identified TopoIV motif was compared with cleavage motifs determined earlier with *in vitro* assays [238,239,257]. Interestingly, the motif resembles more motifs identified for *S. pneumoniae* TopoIV than one identified for *E. coli* enzyme (Figure 26G). Motifs for *S. pneumoniae* TopoIV were identified using drugs (ciprofloxacin and gemifloxacin - Gfx) and with Ca^{2+} (a method considered more native), therefore, nucleotides determined with Topo-Seq likely reflect native binding preferences of the enzyme. Interestingly, identified TopoIV motif resembles much of the *S. pneumoniae* gyrase (identified using gemifloxacin), indicating that the pattern can be conserved for type-II topoisomerases.”