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Opinion on the PhD thesis by Marta Gross

Marta Gross has presented a PhD thesis on "The analysis of the stability of the *Escherichia coli* DnaA protein in the regulation of DNA replication". In this area, she has already co-authored three publications that I am aware of, in PNAS (2015), Frontiers in Molecular Biosciences (2017) and the Journal of Biological Chemistry (also 2017). The main body of the work of her PhD, however, does not appear to have been published yet, and should hopefully earn her a first author paper in a good or even excellent journal.

The question that Marta Gross has addressed under the supervision of Professor Igor Konieczny and Dr Katarzyna Wegrzyn is more specialized than the title suggests. The role of DnaA in bacterial DNA replication is a very classical topic addressed at least since the time of Arthur Kornberg. The novelty of the current work lies in the characterization of this process specifically under conditions of stress.

The main conclusion from the work is that under stress conditions and (p)ppGpp induction, Lon degrades DnaA in a PolyP dependent manner, and thus inhibits DNA replication. The key conclusions are nicely summarized in the paragraph titles. I quote:

- *E. coli* DnaA is degraded during the stringent response
- DnaA molecules resistant to proteolysis bind to the dnaA promoter sequence
- DnaA directly interacts with PolyP via the DNA binding domain
- PolyP and DNA occupy different interaction sites within Lon
- Lon degrades DnaA to arrest DNA replication during the stringent response
- The nucleotide state of DnaA affects its stability during the stringent response

The main conclusion from the work is that under stress conditions and (p)ppGpp induction, Lon degrades DnaA in a PolyP dependent manner. This represents the discovery of a novel pathway for control of DNA replication under stress conditions.

Technical comments

- (p)ppGpp is not well described by the term guanosine tetra- or pentaphosphate. This suggests a linear polyphosphate chain. The term guanosine-3′-5′-bispyrophosphate for ppGpp makes the chemical structure much clearer. Likewise, pppGpp would better be described as guanosine-3′-pyro-5′-triphosphate, making the chemical structure immediately clear. However, the cited terms are frequently found in the literature, and hence this comment is not a criticism of the candidate's work.

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- Is the length or length distribution of the polyphosphate (PolyP) used in the work known? Also, is the PolyP a linear chain, or are branches allowed? My understanding is that "natural" PolyP is linear, but I am not sure about chemically made PolyP. What was used, and what is known about the preparation used in the experiments?
- p. 61, Fig. 12, "starvation" by SHX addition. If I understand the mechanism of SHX correctly, it inhibits Ser-tRNA charging. This creates an imbalance of transferrable amino acids, rather than an overall shortage. In minimal media, it should be possible to create more typical starvation, where all amino acids are in short supply. Was this tested, and if not, why not? Induction of the stringent response upon reaching stationary phase in rich media is likely not due to starvation, but simply a physiological adaptation to avoid bacterial overgrowth.
- p. 63, "Nucleoprotein complexed immunoprecipitation", for my education, how is this different from a standard ChIP experiment probing a single locus only? "Bacterial chromatin" seems to be commonly used, so I don't see a problem with the "ChIP" terminology. What am I missing?
- p. 65, Fig. 15, helicase loading assay: it would have been nice to show as a control that all components in the reaction mix are necessary. For my understanding, not as a criticism of the work: Why is there no smearing of OC form towards high molecular mass observed in panel D, whereas it is seen in panel B? Would such smearing be observed when components of the reaction mix are omitted?
- p. 66, Fig. 16, PolyP binding to DnaA: Marta Gross finds that DnaA binds PolyP, whereas BSA and DnaB do not. This could have a trivial physical reason. According to UniProt, DnaA is a very basic protein (calculated pKa of about 8.8). In contrast, BSA is slightly acidic and DnaB strongly acidic (surprisingly, calculated pKa of 4.9). As PolyP is strongly acidic, binding to DnaA, but not BSA or DnaB could simply be a pKa effect. A control of a strongly basic protein NOT binding PolyP would have made this part more convincing. To Marta Gross's defence, the denaturation control adds to her case for specific binding of PolyP to DnaA. Moreover, the (in my eyes) most plausible mechanistic model to interpret Marta Gross' data also supports the conclusion.
- p. 67, PolyP binding to Lon: Marta Gross concludes from PolyP binding, but not DNA binding to Lon mutants with K→E and R→E substitutions that PolyP does not bind to the DNA binding site of Lon. Upon first reading of the thesis, this conclusion appeared to strong to me. It is also possible that the new glutamates repel the negatively charged phosphodiester backbone of DNA. A bulky molecule such as DNA may not be able to evade this repellence, but for (presumably smaller, see a previous question) PolyP, this may not be the case. I have to acknowledge, however, that my doubts on this point may be unduly tinged by my favourite mechanistic

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interpretation of the data in terms of a *physical* PolyP tether between Lon and DnaA, which is at this point highly speculative (and may well not be right).

- p. 75, DnaA-ATP is resistant to proteolysis. As pointed out in the thesis, for this experiment, the nucleotide states of DnaA and Lon have to be controlled independently. This is challenging, the reconstitution of the RIDA system to achieve it deserves a compliment! Since the ATP-form is resistant and the ADP form of DnaA is degraded, it is clear that the nucleotide states of DnaA and Lon have been successfully separated (ADP should be an inhibitor of Lon).

Editorial comments

The thesis is conventionally structured.

- The introduction is very interesting to read. It strikes a good balance between presenting the classical picture of initiation of bacterial DNA replication, and the presentation of development in recent years, that –at least in my view– have sometimes more confused than clarified the overall picture (e.g. with the finding that there are three not two active polymerases in the bacterial replisome, or with the finding that not only lagging strand synthesis, but also leading strand synthesis, is discontinuous).

- The abstract section provides a clear statement of the research question, and concisely summarizes the results.

- The materials and methods section is comprehensive, and reports on methods for a very extensive experimental program. The number of proteins that were expressed and purified, and of protein complexes that were reconstituted, is impressive. I have one suggestion: for a reader who is not an expert in the field, it would be nice to learn which purification protocols are novel, and which have been adapted from prior literature. Additional references here would be helpful. I also encountered a widely off statement about antibiotic concentrations (p. 36), presumably due to a confusion of stock concentrations and culture media concentrations.

- The results section is the strongest part of the thesis. Here, Marta Gross presents a clear and logical outline of her work. The diversity of reconstitutions and assays that she is made to work is very nice. For someone only working in the broader field, additional controls in this part would have been helpful (see the comments on merit). Otherwise, this part is very reasonable, and pleasant to read.

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The discussion is very careful not to over-interpret the data. As a reader with own background in mechanistic biochemistry, I would have been tempted to suggest a mechanistic model for how PolyP mediates DnaA degradation. On reflection, I have to agree with Marta Gross that the data are not yet sufficient, and that it is prudent to keep an open mind about the exact mechanistic basis of her observations.

In an overall well written thesis, I found a few minor glitches.

p. 16 "there can be distinguish"
p.19 "which was deduced basing on the crystal structure"
p.21 "Eukatiotic" where "Eukaryotic" is meant
p. 22 "Basing on" where "Based on" is meant
p. 26 "The study, with the use synchronized E. coli cells"
p 54, "The pellet, arised from centrifugation"
p. 60, "were spinned"
p. 64, "retardation in bands migration"
p. 64, helicase loading assay: error in method references, method 18, not 19
p. 64, "retardation in bands migration"
p. 67, "obtained results". For comparison, what are "non-obtained" results?
p. 68, "further experiments are at demand" should be "further experiments are required"

A glitch has also occurred in the bibliography, where many citations lack page numbers, presumably due to a problem with the reference manager.

Potential implications

While the biochemical model is clear, it leaves the mechanistic interpretation open. Is PolyP acting as a physical tether between DnaA and Lon? Is ATP/ADP ratio altered under stress? While it is reasonable to refrain from such speculation in the absence of concrete experiments, it would be interesting to hear Marta Gross' opinion on the likely mechanistic basis of the pathway that she has discovered.

Conclusion

Overall, the thesis contains a very vast amount of biochemical work, with an impressive number of reconstitution experiments and assays that have led to the discovery of a novel pathway. There is no doubt that the work is **up to the standards of a PhD thesis and that Marta Gross deserves the award of a PhD title**. The committee may also consider awarding a distinction.

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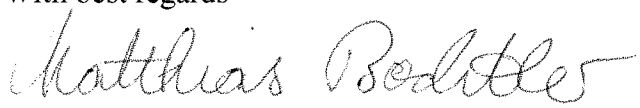
With best regards



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