Introduction to the Revised Application

We thank the reviewers for their insightful comments and criticisms; we have taken great care to revise our application to address their concerns. Among these we accept the reviewers' recommendation that the project use the multi-PI format given that each brings a high level of expertise and commitment to this project.

It is clear that, whereas the panel finds our proposed mechanism for DNA packaging compelling and tractable, there was concern that: 1) the mechanism is so well supported that further verification would represent only incremental progress; and 2) that if our proposed mechanism is incorrect, the new work will have been done in vain. We believe that both concerns result at least partially from the organization of the proposal. First, Aims were previously centered around biological questions. As a result of using disparate approaches to address each question, the text was often confusing regarding what had been done and what would be done. We have thus reorganized the Aims around experimental approach; we believe the revised text provides a clearer explanation of the proposed work. Second, we failed to properly articulate: 1) uncertainties associated with both our and other models; and 2) how the proposed work can generate insight into *any* model. For example, although comprehensive, the proposed helix-to-planar mechanism is the product of limited induction. Thus, it requires experimental verification at several levels, including: 1) the proposed quaternary helical to planar transitions; 2) the molecular basis of force generation that drives these transitions; and 3) how coordinated nucleotide binding in individual subunits actuates this force generation and DNA translocation.

In the revised submission, we point out that our mechanism is largely based on the cryoEM structure of a single endpoint of the mechanochemical cycle: the helical structure of the ATP-bound motor. Hence, proving or disproving our mechanism would thus require visualizing additional structural states. In particular, to evaluate the validity of the helical to planar mechanism, it is necessary to image the other endpoint of our mechanism, *i.e.* the proposed planar state adopted at the end of the burst when all subunits bind ADP. Indeed, without this structure, alternative proposed ASCE mechanisms, such as the hand-over-hand and helical treadmilling, could also partly explain our helical structure. Therefore, we introduce these and other mechanistic possibilities, and emphasize how the proposed work will help distinguish between mechanisms as well as suggest new mechanisms should the data point us in that direction. Further, we also explain that while the ADP-bound structure will greatly help discriminate between the gross quaternary transitions in various mechanisms, it will not confirm or refute the proposed basis of force generation driving transitions in any mechanism. We now clarify how imaging additional structures, including DNA-free structures, are necessary to evaluate the proposed mechanism of force generation during packaging. Similarly, proposed single molecule experiments are also necessary to evaluate both quaternary changes that occur during packaging (via single molecule fleezers experiments to measure DNA rotation in Aim 2.2 and subunit stepping in Aim 2.3) as well as how nucleotide binding induces the forces that drive the resulting structural transitions leading to DNA translocation (mutant experiments in Aim 2.1). Importantly, in the revised proposal, we clarify that the proposed experiments are not limited to proving or disproving our mechanism; they also inform other mechanistic possibilities equally well. Hence, in the revised text we not only clarify the considerable need for experimental validation of our proposed mechanism, we also address concerns raised regarding what happens if our mechanism is wrong.

Another concern raised in the discussion was that insufficient detail was provided to evaluate experimental feasibility. Upon re-reading our previous proposal, we can understand how reviewers arrived at this concern. In particular, because of limited space, we expected an unrealistic level of familiarity with our previous work. Hence, we have taken care to both provide additional experimental detail, and to more clearly reference prior work that contains relevant protocols and demonstrates that the proposed experiments are doable. For example, the revised text explains how valuable mechanistic information can be derived from seemingly intractable data generated from various mixtures of mutants in motor rings (Aim 2.1). We also describe how we can interpret complex FRET signals arising from multiple FRET acceptors to extract meaningful information in the molecular fleezers experiments proposed in aim 2.3. Similarly, for aims 2.2 and 2.3, both the novel optical setups and reagent preparations are more thoroughly described. For Aim 1, there were questions regarding how/whether DNA packaging samples are assembled on grids, and whether assembly timescales are compatible with the proposed cryoEM experiments. We now explain that proposed structures of motors assembled on procapsids are derivatives of the type of intermediates and structural analysis we have published in the past; indeed grid preparation and imaging procedures are published in the cited works. There was also some confusion regarding imaging motors in the absence of DNA and isolated motor rings. Thus, we have revised the text to clarify that the former can provide information about force generation that requires imaging in the absence of DNA, and that the latter can provide information about the energetic landscapes of motors that would be difficult to obtain from motors assembled on procapsids packaging DNA.