Response to Reviewers

We would like to thank both reviewers for their helpful and constructive comments. In response to these comments, we have revised the manuscript extensively. Most importantly, we have now added free-energy differences calculated using FEP for all complexes considered, and we show that these values correlate strongly with the max force obtained under SMD. Below follows a detailed response to all comments.

Reviewer 1 (Ilan Samish)

1. I don't know the word limit of the title but the journal editor should consider changing the paper's editor to reflect the fact that a single case-study (GPI-hTfR1) is analyzed rather than the general phenomenon of virus-receptor binding. This is needed especially as different case-studies often produce different results. Indeed, the authors well articulate already in the abstract and then in the introduction the weak spots of the field. In the same line of thought, the case-study differences and the analyzed data is not only b/c of PPI problems. An important paper in the field that caused David Baker to work hard on the problem is http://www.ncbi.nlm.nih.gov/pubmed/?term=19561092 where it shown the mutation prediction is good on average but not in each case-study. Actually, the authors may want to refer to this paper in their mutation analysis. Likewise, steered MD was already used for PPI e.g. Cuendet M, Michielin O. 2008 which the authors cite.

We agree that the title was misleading. We have changed it to the following to reflect to scope of the manuscript: “Analyzing Machupo virus-receptor binding by molecular dynamics simulation”

2. The authors imply that their methodology is new. Yet, steered MD is a common methods and was used also for mutations e.g. pubmed I (PMID: 23836247). The relevant statement should be softened.

Thanks for pointing us to the referenced paper. We were collecting data and finishing up writing this manuscript before that paper was published so we missed it. We now cite this paper, and we have softened our language of the entire paragraph to:

> “Here we propose that much of these complexities can be avoided if all we are interested in is a relative comparison of the effects of different mutations on protein-protein interactions, rather than measuring an absolute or relative binding affinity with experimentally realistic units. We impart a pulling force within an all-atom molecular dynamics simulation on one member of the complex while the other is held in place. Then, we measure the force required for dissociation (Lu and Schulten 1999; Isralewitz et al. 2001b,a; Park and Schulten 2004; Gumbart et al. 2012; Mino et al. 2013). Although such biasing techniques are commonly used in protein-ligand binding problems, they are less commonly applied to protein-protein interactions, and almost never to mutational analysis in a protein-protein system. This is largely the result of free energy convergence difficulties and computational limitations (Cuendet and Michielin 2008; Cuendet and Zoete 2011). Using a proxy for relative binding affinity rather than caluclating absolute affinities can solve these problems. Here, as proxies, we use the maximum applied force required for separation and the area under the force-versus-distance curve (AUC). For comparison, we also calculate relative free energy differences using the traditional dual topology FEP paradigm, and we show that the two approaches yield congruent results.”

In addition we removed this misleading sentence:

> "By extending the SMD technique to investigate a protein--protein interaction we have, in many ways, changed the scope of its application."

3. The spring constant was set to 5 kcal/mol/Å and the velocity set to 1 Å/ns. This choice of parameters should be discussed in light of other SMD publications as are the other choices within the SMD protocol. In this discussion, differences should be highlighted and explained.

We have added some language to that effect in the methods. In the field of force biasing simulations it seems both of these parameters are chosen on a more pragmatic than a theoretical basis. Even when one is calculating rigorous free energy differences (which we are not doing here), the force constant is chosen based on a limited set of test runs. This is probably because there is no reason to believe free energy calculations should be sensitive to the choice of biasing parameters. In several papers, Gumbart et. al. go to get lengths to show that the final calculations are not even sensitive to the free energy method chosen, not to mention the biasing parameters. (Gumbart J C, Roux B, Chipot C. 2013b. Efficient determination of protein-protein standard binding free energies from first principles. J. Chem. Theory Comput. 9:3780–3798.)

4. in section 3.2 you write: "For SMD, at the centroid of 176 several atoms a force was applied to a single point in GP1." Readers should be able to generally repeat the simulation and actually the choice of the atoms to be pulled may affect results. Thus, the list of pulled atoms should be attached as a supp material.

We intended to upload the namd input parameters, but it was overlooked in the final submission. We now provide our set-up files and computational pipeline in a github repository, https://github.com/clauswilke/MACV_SMD. In addition, we greatly expanded the steered molecular dynamics section in the methods to include the requested information. Perhaps it was less than clear, but the pulled atoms were already listed in the previous submission. In NAMD one can simply specify the CA atoms by listing the residue number and requesting only CA's. We have clarified it in the following way:

> "The center of mass of the α-carbons of all residues (163-318 in linear numbering) in GP1 received an applied force during the simulation. The NAMD convention does not actually apply a force to all α-carbon atoms but rather uses the selection to compute an initial center of mass. Then, during the steering run, the single center of mass point is pulled with the parameters described below."

Reviewer 2 (Matteo Masetti)

1. Methods are not enough detailed.
Why did the authors use a steering force acting on all alpha carbon instead of the more common choice of either steering just one atom or COM of one of the two counterparts? Are the authors using the protocol introduced by Cuendet and Michielin?
If so, this must be clearly stated, otherwise much more details should be reported in the Methods section regarding the steering procedure.
Moreover, certain methodological details are reported in the Results (see page 8, rows 176, 177), increasing the difficulty in understanding the steered MD procedure.

We agree with these comments and we have addressed them in the revised manuscript. See also our

response to points 3 and 4 of Reviewer 1. We also removed the sentences containing details about methods from the Results section and added the relevant information to the Methods section.

2. In the Results, at page 7 (rows 140 to 143), the authors state that large structural rearrangements of an interfacial flexible loop are expected upon binding, and they claim that because of this traditional computational tools are of limited usefulness. While one might agree with this sentence, the authors seem to overlook the fact that such a rearrangement complicate any other simulative strategy as well.
How does this loop behave during steered MD simulations?
Does it unfolds or it stays in place?
A more detailed structural analysis is required.

We agree. In principle, flexibility would complicate any simulation study, but probing such rearrangements would really be impossible for a technique like MM/PBSA or FEP. At least with SMD (or a few related biasing techniques) the computational overhead to achieve such rearrangements does not increase substantially as the number of mutations increases, while it does in FEP. Regarding the requested analysis of structural rearrangements, see our answer to the next point.

3. As a follow up of the previous point, where are the mutations located relatively to the protein-protein interface?
Are they located in the flexible loop? In this case, how do the complexes "react" to the mutations?
Are there major rearrangements or just local motions during the production runs preceeding the pulling phase?
Very basic analysis such as computing the RMSD over time and RMSF per residue should be performed and reported.
Moreover, showing the location of these mutations in Figure 1 would improve the comprehension of the manuscript.

All of the mutations are within 5 Angstroms of the interface. We have now stated that explicitly here:

> "In total, we tested 7 point mutants and 3 double mutants in addition to the WT complex (Table 1 and Figure 7). All of the mutations are within 5 Å of the protein-protein interface."

There are no major rearrangements during equilibration. We have included plots of the RMSF versus index and the RMSD over the equilibration trajectories (Figures 2, 3 in the revised manuscript). They show the flexible loop on the free transferrin receptor is quite immobile as part of a beta-sheet with the virus bound. There are two flexible sites in that loop when the virus is bound, but they are highly solvent exposed, not involved in the binding interaction, and would be expected to be fairly mobile. According to the added RMSF plots, after viral unbinding, the loop appears to become quite flexible (Figure 4 in the revised manuscript). Its mobility shift appears to be greater than that of any other part of the receptor. As a result we added the following text:

> "Despite the fact that viral binding occurs at the site of a flexible loop in the free hTfR structure, our data shows after binding the strand is extremely rigid. In the bound conformation, only two sites of the loop have root mean squared fluctuation (RMSF) values in the top half of all receptor sites during equilibration (Figure 2), and those are almost completely exposed to solvent. This is unsurprising considering the high degree of burial that occurs as a result of viral binding. Computing the root mean squared deviation (RMSD) of the entire structure over the trajectory shows that none of the mutations are so deleterious as the cause rapid unbinding. In fact, the RMSD over trajectory looks highly invariant across mutants (Figure 3). In the unbound state, calculated near the end of the SMD trajectory, all of the residues in the WT receptor interfacial strand are in the top half

of RMSF over all receptor sites (Figure 4). Thus, if sufficient simulation time is not dedicated to allowing the unfolding process to occur, standard free energy techniques may miss the energetic contributions that result from ordering the flexible loop in the hTfR apical domain."

4. I acknowledge the authors that probing the viability of the approach to point mutations before moving to more challenging cases is a good practice, and I also appreciate the fact that they do not overemphasize their results.
However, if I properly understood, the only experimental validation of the protocol is provided by three point mutations which returned two statistically insignificant correct results (N348K, vR111A) and one statistically significant wrong result (Y211T).
Unfortunately, this is not enough to support the steered MD protocol.
I suggest the authors to enrich the paper in either one of the two following ways:
1. by performing a detailed comparative analysis of the interactions and their changes upon mutations along the steereing phase (both from a structural and energetic point of view, e.g. by calculating the interaction energies as reported in the Cuendet and Michielin paper, by monitoring hydrogen bonds braking/formation and so on),
OR
2. to compare the steered MD results for the three alanine point mutations with more established free energy approaches (FEP or TI, see for example the recent paper by Luitz and Zacharias, Proteins 2013 81:461).

We agree with this comment, and in response we have performed dual topology FEP on all of the same mutations. That data is now included in Table 2 and the correlation between SMD max force and FEP dG is plotted in Figure 6. We have integrated FEP language throughout the paper. In addition, we have included the following text:

"We proceeded to compare our SMD results to that of the standard dual topology FEP approach to calculate relative free energy differences. The correlation between the energetically rigorous FEP and our statistical approach is high. For all 11 complexes tested, the correlation between max force and FEP was r=-0.795 at p=0.0034 (Figure 6), and the correlation between AUC and FEP was r=-0.593 at p=0.055. Because of the strong correlation, we refer exclusively to the SMD results for the remainder of this work, focusing primarily on max force."

5. In the Introduction, at page 1, (rows 14 to 17), the authors state that methods such as FEP and TI rely on a two state model, "with no intermediate steps". This is in a best case scenario a misleading sentence, since FEP and TI are computational frameworks that, in order to return meaningful results, in most of cases DO NEED intermediate steps. In fact, the sentence is also misleading since the authors refer to these methods as to "two state models" whereas they are generally known as "pathway methods" to be easily distinguished by "endpoint methods" such as MM-PBSA, LIE, ..., where only ensemble averages of certain endpoint properties are used without intermediate states!
This source of confusion must be properly addressed.

We apologize, our phrasing was not sufficiently clear. We meant to imply that large ending state transitions become computational intractable, and as a result, large changes can require a large number of intermediate (and expensive) calculations. The section now reads.

"By contrast, first principles methods can forgo training, but currently available methods such as free energy perturbation (FEP) and thermodynamic integration (TI) rely on a

transitional model (where one state may be wild-type and the other may be a mutant) to make rigorous free energy calculations (Gilson et al. 1997; Lu et al. 2004; Chodera et al. 2011; Gumbart et al. 2013a). While these may be considered two of the gold standard techniques for calculating affinity differences, there are a huge number of theoretical and technical complexities that must all be properly managed to ensure a converged solution (Gumbart et al. 2013b). Such considerations quickly come to dominate the protocol, and the necessary book keeping introduces the possibility of human error (Gumbart et al. 2013b). Moreover, as the two ending states look ever more dissimilar the chances of convergence fall rapidly. To ensure convergence, these techniques are typically limited to small differences (such as point mutant comparisons) with a few, very impressive exceptions (Wang et al. 2006; Gumbart et al. 2013a,b). For most investigators, larger differences quickly become intractable as the number of intermediate steps required to compute a converged solution grows or the complexity of adding restraining potentials and computing approximations expands (Wang et al. 2006; Gumbart et al. 2013a,b).”

6. In the Introduction, at page 1 (row 20), the authors refer to the use of steered MD in the prediction of relative binding affinity between wild type and mutant protein-protein complexes as a "new method". This is an overrated sentence for two reasons: 1. the authors show a procedure, a protocol (if it was properly detailed), they do not develop any new methods; 2. a similar approach has already been reported by Cuendet and Michielin as early as 2008, as the authors properly acknowledge in the Results section. The sentence should be tuned down, and this also holds for the sentence at page 11 (rows 266, 267) where the auhtors state that they changed the scope of application of SMD simulations.

Agreed. See point 2 of reviewer 1.

7. Page 4, row 71. Is the system size really of about 28,000 atoms? Systems of about 30,000 atoms are relatively small, whereas by looking at Figure 1B I would have expected at least 50,000 atoms. Please double check.

The system is indeed about 28,000 atoms (actually between 28,000 and 28,100 depending on which mutant). We used a 5 Å buffer around the bound complex and left a 20 Å buffer in the pulling direction.

8. Page 4, rows 74, 75: "This was accomplished by setting beta=1." What do the authors mean? What does the beta parameter stand for?

We meant the B-factor column in the pdb. In VMD the B-factor column is called beta. The sentence now reads:

“In addition to the modeled system, for equilibration we generated a configuration file that fixed the α-carbon backbone. This was accomplished by setting the B-factor column to 1 for the fixed atoms and to zero for all other atoms.”

9. Page 4, row 84. The authors state that the exact input file is reported in the supplement. I wasn't able to find any input file in the supplement, but I apologize beforehand with the authors in case the input file was actually provided.
Please double check. Anyway, the authors should at least indicate in the main text very important simulation parameters such as the temperature control method employed and the cutoff radii for nonbonded interactions.

Apologies. It was an oversight in the final submission. They are now provided as part of the github repository we made for this project (https://github.com/clauswilke/MACV_SMD).

10. Page 6, rows 131, 132. Please provide some more details regarding the "pairwise.t.test function" and the "FDR p-value correction".

We now write:

> "We tested for signifiant differences in maximum force or AUC by carrying out t tests for all pairwise combinations (each mutant compared to each other mutant), using the pairwise.t.test() function in R. We adjusted p values to correct for multiple testing using the False-Discovery-Rate (FDR) method (Benjamini and Hochberg, 1995)."

11. Page 8, row 171. Please avoid odd sentences such as "we generated a physically realistic simulation world", as this is what people usually do when performing Molecular Simulations, there is no need for such an emphasys in a scientific paper.

The sentence was removed.

12. It seems to me that the authors have a menageable amount of data. Just showing a force profile (Fig. 2) and the maximum force probability distribution (Fig. 3) for selected entries in the main text is ok, but the authors should also show all the force profiles and maximum force probability distributions for all the system they simulated in the supplement.

All force and distance data are provided in the github repository, as are figures of average force profiles for each mutant in the figures/force_curves folder. In the manuscript, we visualize the maximum force probability distribution for all mutants via box plots in Figure 8.

13. Page 13, row 321. What do the auhtors mean with "unrestrained SMD"?

We have removed the word 'unrestrained'.