The switching mechanism of the bacterial rotary motor combines tight regulation with inherent flexibility

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Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information. Based on these comments, I am afraid that we had to conclude that the study is not a sufficiently strong candidate for publication in The EMBO Journal.

As you will see, while reviewer #3 is positive in their assessment, reviewers #1 and #2 raise numerous substantive concerns regarding the conclusiveness and interpretation of data, and they are not convinced that sufficient support to the proposed model of stepwise activation of bacterial motor switching is provided. Given these opinions from good experts in the field, I am afraid that we cannot offer further proceedings towards publication of your manuscript in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I regret that I cannot communicate more positive news, but I nevertheless hope that you will find the comments of our reviewers helpful.
Referee #1:

Afanzar and co-workers have attempted to identify the role of a specific domain of FliM - a core component of the flagellar switch - in the reversals of the flagellar motor. The authors claim that the motor switch is flexible and that this flexibility helps with cell navigation. They have further attempted to glean information regarding two related attributes of the switch, its ultrasensitive switching and CheY acetylation.

Major concerns

'Flexibility' is a bit of a misnomer since all polymeric complexes exhibit flexibility of varying degrees. Nonetheless, the data is extensive and some of the experiments are interesting. However, I found it challenging to make sense of the proceedings. Several of my reservations stemmed from the lack of clarity in the description of the results. The parts that I did enjoy reading appeared to provide incremental information; the impact wasn't obvious to me. The term 'biphasic' and 'monophasic' have not been described. Presumably the authors didn't consider these to be important. However, large sections of the results have been dedicated to these minor effects. The authors strain to establish the relevance of short-lived switching to motility but it was not very clear whether it really influenced directional persistence. Some of my broader comments are listed:

A large part of the FRET responses observed in Fig 1B is likely due to the interactions between the thousands of cytoplasmic FliM and CheY-P molecules; they are in abundance compared to a few dozen FliM units within individual switches. Therefore, the FRET response is unlikely to be a good indicator of the nature of interactions between the mutant switch and CheY-P. The appearance of an attractant response in the strain missing the kinase (negative control) is troubling. I wonder if the same complex phenomenon was responsible for the apparent weak attractant response in the tethered cell assay (Fig1D), in which case none of the experiments in Fig 1 really show an effect at the level of the flagellar switch. Further, overexpression of CheY is necessary to induce switching in the FliM_dN motors, and yet no repellent response was observed when treated with 1 mM Leucine (Fig 1E). This is very strange considering that the switch is ultrasensitive. A representative speed trace would have been more instructive here rather than the tethered cell depiction (Fig 1C).

I believe that a simpler interpretation of the results could be considered. Perhaps, the overexpression of CheY partially compensates for the lack of the N-terminus of FliM by promoting nonspecific interactions within the switch. These non-specific interactions may not be relevant under wild-type expression levels of CheY.

It is not clearly explained by the authors what they meant by biphasic response in Fig 2B (page 5). I look at Fig 2B and I fail to see such a response. The authors follow up with the term 'biphasic trajectory' - what does that mean?

What do the authors mean by relaxation of the motor from CW to CCW; how could the kinetics of relaxation be possibly determined by the value of equilibrium constants as they claim on page 5?

What is meant by monophasic response to acetate removal? At some point the authors decided to alter the meaning of bi- and monophasic responses from steady-state phenomena - Fig 2B purportedly demonstrates a biphasic response that depends on IPTG concentrations and not time - to kinetic phenomena (Fig 2D). This is difficult to follow especially since biphasic, monophasic, slow and fast phases appear throughout the manuscript and discussions.
The authors have tried to establish some degree of relevance to these obscure effects by analyzing directional persistence (page 12). The section is unconvincing since 'angular shift' a key attribute has been defined as 'not tumbling behavior' - this could mean anything including loss of motility. The term is employed only once and has probably been superseded by 'angular deflection', 'turning angles' and so on in later instances. Like several other places in the text, the description in this section is scientifically imprecise. The authors claim that they subtracted 'FliM_N-CheY measurements from the delta cheY measurements' - I don't understand how measurements can be subtracted from each other - and then they find that the subtracted value peaks at 20 C (Fig 7C). Assuming that the probabilities are being discussed, it is unclear how meaningful, positive probability values were obtained by subtracting higher values (FliM_N-CheY) from lower values (delta CheY).

Overall, large parts of the manuscript are unwieldy and impenetrable; some of the claims that I could track appear questionable at best.

Referee #2:

The manuscript supplied a large amount of data on behavior of the bacterial flagellar switch in a FliM_ΔN strain, which led to interesting possible mechanism for motor switching, especially for the function of the two weak binding sites (FliN and FliM_M) of CheY-P. However, many places in the manuscript need clarification, some conclusions in the manuscript are too strong or incorrect, and many conclusions are inconsistent with one another. Please see below for comments:

In the last paragraph of result 1: Different strains were used in Fig. 1E & F, please clarify the difference in the main text. In the last sentence: "These results suggest ... that acetylation is apparently more potent than phosphorylation for this." This conclusion is incorrect, as there is barely any change in CheY-P level in Fig. 1E for a cheZ-deletion strain with repellent stimulation, whereas the change in CheY-Ac level is significant in Fig. 1F.

In Fig. 2A, the binding affinity of CheY-P and CheY(D13K) to the motor are significantly different, please comment on the reason.

Near the end of Page 5: "This difference between the responses of FliM_N-CheY- and CheY expressing strains to acetate removal is consistent with the function of FliM_N as CheY activator, affecting the binding stability of the latter to the motor." Apparently from Fig. 2A the binding affinities for FliM_N-CheY and CheY to the motor are the same, please clarify the inconsistency.
In Fig. S4, why do benzoate and leucine induce an attractant-like response, whereas in Fig. 2C benzoate generate no response? Moreover, why the acetate-removal response in Fig. S4 (only slow decay) is different from those in Fig. 2D (fast decay or fast + slow decay)? How fast was the rate of addition or removal of the stimuli?

Near the end of the result section "CheY binding to the motor is biphasic": "we only measured spots of high intensity FliMΔN-YPet signal." Judging from Fig. 2B, it was impossible to measure motor spots. Since most of the fluorescence came from cytoplasm, it would be more convincing to look at signals from single motor instead of single cell. A TIRF measurement may be necessary.

Also near the end of the result section "CheY binding to the motor is biphasic": "This is because clockwise rotation could be observed at CheY concentrations lower than those of the cooperative phase". This is inconsistent with what was claimed in result 1, where 100 μM CheY was needed in a cheZ-deletion strain with FliM_ΔN to observe CW rotation, which was larger than the Kd of the cooperative phase. And probably more CheY-mCherry is needed due to the possible interference of mCherry. Moreover, the Kd for the non-cooperative phase was about 11 μM, which is similar to the value of 15 μM for CheY-mCherry binding to FliM_wt as said in result 1. So how the conclusion was reached that the non-cooperative phase was due to binding to FliN (a weak binding site)?

In Fig. 4A, at low or high CW bias, the survival probability was claimed to be a single exponential. Then in Fig. 4B, how were the data at low CW bias for the second phase and the data at high CW bias for the first phase obtained?

In Fig. 4D & Fig. S5, the peak reversal frequency (even with FliM_N fused to CheY) is about 8-fold higher than the wild-type cells measured in ref. 34. And the biphasic decay for the survival probability was different from that observed in previous studies. However, the reversal frequency and the survival probability are sensitive to the analysis algorithm. A control experiment using a wild-type strain should be performed using the same data-analysis procedure, instead of just quoting the value from ref. 34.

In Fig. 4G, the FliN(A93D) mutant that impaired CheY binding still exhibited biphasic decay. This was inconsistent with the claim that the fast decay was induced by CheY binding to FliN.

If the claim was correct that the fast phase in CW interval distribution was due to binding to FliN, and the slow phase was due to binding to FliM_M, the lengths of those intervals were determined by the off rates for these two binding sites. If the FliM(E214W) mutation increased the affinity of FliM_M for CheY as claimed, probably by decreasing the off rate, the slow phase should be even slower so that the separation between the fast & slow phase should be more obvious. This is in contradiction to what was observed in Fig. 5I.

On page 11 line 1-2: The conclusion was too strong. The extension of CheY dwell time at the switch by acetylation does not necessary mean FliM_M has a preference for CheY-Ac. It only means that acetylation increases the CheY affinity.
The dwell time distributions (Fig. 6B-E) were obtained with wild-type motors. The effects of phosphorylation and acetylation on the two decay rates did not "suggest that the first and second clockwise phases are due to CheY interaction with FliN and FliM_M, respectively, and that, apparently, CheY-P and CheY-Ac preferentially bind to the former and latter, respectively." (page 11, 3rd paragraph). As the authors suggested in the introduction, CheY binding to FliM_N dominates over FliN and FliM_M with the latter two being weak binding sites.

The claim above was also inconsistent with Fig. 3D and the conclusion at the end of the first paragraph on page 6, where CheY-P binding to the switch showed two phases, presumably one is binding to FliN and the other to FliM_M.

Fig. 3D and Fig. 6H are inconsistent, with Fig. 3D showing two phases and Fig. 6H showing single phase. And the two phases in Fig. 3D were obtained with CheY-P, without involving CheY-Ac.

The distribution of turning angles for swimming cells (Fig. 7B) depends sensitively on how the turning points were determined, especially when there were large amount of small turning angles. Please provide details on how the turning points were determined.

The meaning of subtraction of two survival probabilities (Fig. 7C) is not straightforward, as the subtraction will be zero at 0 deg and large angles with a peak in between. I would suggest using the probability density distributions.

Last paragraph on page 13: "The observation that CheY binding to the switch is non-cooperative6,7 ...". More recent measurement (Fukuoka et al, Science signaling 2014, 7:ra32) actually demonstrated with single motor fluorescence that the binding is cooperative, which agrees with that modeled by Duke et al (JMB 2001, 308:541, Fig. 6). Please cite and comment on this.

The kinetic model of the switch is confusing (Page 14). Please provide more details. I do not understand the purpose of the model. Was it to explain the ultrasensitivity of the response of the wild-type motor (ref. 5)? Non-cooperative in the response of a FliM_ΔN stain (Fig. 2A)? Or the ultrasensitivity of CheY-P binding? But the authors already claimed that the binding is non-cooperative by citing ref 6&7. I think Fig. 3D is probably worth to be explained by a model. Moreover, why assuming a single FliN-binding site and two FliM_M-binding sites?

Referee #3:

The bacterial flagellar motor of Escherichia coli is a bi-directional rotary motor powered by proton motive force across the cytoplasmic membrane. The flagellar motor has a directional switching device that allows the motor to spin in both counterclockwise (CCW) and clockwise (CW) directions. When all motors rotate CCW, E. coli cells can swim straight in liquid. When multiple motors spin in the CW direction, E coli cells stop swimming and changes their swimming direction. Three flagellar proteins named FliG, FliM and FliN form a cytoplasmic ring complex called the C ring that acts not only as part of a rotor of the flagellar motor but also as the directional switching device. The flagellar motor is placed under a control of a sensory signal transduction pathway and E. coli cells sense temporal changes in environmental stimuli to migrate towards more suitable conditions. The chemotaxis signaling protein CheY binds to FliM and FliN in the C ring and so the
motor switches its rotational direction from CCW to CW. However, it remains unknown how the CheY-binding to FliM and FliN induces conformational arrangements of the C ring structure responsible for directional switching. In the present study, the authors have developed high-resolution single molecule imaging techniques combined with various genetic modifications to measure the interaction between CheY and the switching device at the flagellar base in a very precise manner and have shown that

1. The binding of CheY to the binding sites of the C ring with low affinity results in CW rotation;  
2. The interaction of CheY with the N-terminal flexible region of FliM (FliMn), which has a very strong binding affinity for CheY, allow CheY to adopt an active conformation to bind to the weak binding sites of the C ring;  
3. The response of the switching device of the motor to CheY is biphasic;  
4. Mutations in FliN and FliMm affect the first and second phase of clockwise generation.  
5. Under their experimental conditions, the phosphorylated form of CheY (CheY-P) predominantly binds to FliN with low affinity, thereby causing a short CW interval whereas the acetylated form of CheY (CheY-Ac) bind to FliMm with high affinity to stabilize the CW state of the flagellar motor, thereby generating a long CW interval.

These results led to a plausible hypothesis that the CheY binding to FliMn allows CheY to bind to FliN with low affinity, thereby inducing conformational rearrangements of FliM to expose the second CheY binding site on the molecular surface of the FliM structure so that CheY to bind to FliMm to stabilize the CW conformation of the C ring in a highly cooperative manner. Overall, the paper is reasonably well organized and clearly written. The methods seem reliable, and the results and conclusion are sound scientifically. This research article would be of great interest to general readership, providing important advancements in our knowledge on directional switching mechanism of the flagellar motor. Although this reviewer thinks that the current form would be suitable for publication in EMBO Journal, this reviewer has only minor comments.

1. Potassium benzoate does not cause CheY acetylation but addition of 10 mM potassium benzoate at external pH 6.5 and pH 6.0 not only increase the switching frequency of the flagellar motor but also causes very long CW intervals, leading to tumbling of Salmonella cells (Sakai et al. mBio 10: e00079-19, 2019). This raises the possibility that the phosphorylated form of CheY can bind to FliMm in a way similar to CheY-Ac when the concentration of CheY-P is high enough even in the absence of CheY-Ac. Therefore, it would be interesting to see whether 10 mM benzoate at external pH 6.5 and 6.0 causes biphasic CheY-binding to the motor in the absence of CheY-Ac.

2. The authors should cite the following paper because this paper is the first report describing the dissociation rate of CheY from the rotating motor in single E. coli cells.  
Fukuoka H, Sagawa T, Inoue Y, Takahashi H, Ishijima A. Direct imaging of intracellular signaling components that regulate bacterial chemotaxis. Sci Signal 7: ra32.
Thank you for letter. After very carefully reading and digesting the reviewers' comments, I felt that I must write to you. This is mainly because it became very clear to us that Referee #1 apparently did not understand the paper. This is obvious (a) from the first paragraph of his/her review, in which he/she wrongly summarizes the manuscript, (b) from his/her own words that "several of my reservations stemmed from the lack of clarity in the description of the results" (third sentence in the second paragraph of his/her review), and (c) from the comments themselves, which clearly point to misunderstanding. The comments of Referee #2 are constructive and excellent. All of them are addressable. Even though we worked very hard to make the original manuscript very clear in spite of being quite elaborated and sophisticated with lots of experimental data, apparently, we did not succeed enough in doing so, as only one of the three reviewers wrote that the paper is clearly written.

The comments of the reviewers unmistakably point to the parts of the manuscript that are insufficiently clear. In view of our conviction that the drawback here is not scientific but rather a problem of insufficiently clear writing, I would like to request your permission to revise the manuscript and to submit to you, when ready, a much clearer version, in which the novelty of the study and the breakthrough in understanding the mechanism of the switch will be obvious to all readers.

As I mentioned in my original letter to the editor, among the known biological switches, the switch of the bacterial flagellar motor has been of special interest. Revealing its molecular mechanism in this study, the unprecedented features of this mechanism, the interest that the switch of the flagellar motor generates combined with its mechanism being enigmatic for so long, and the wide prevalence of biological switches, potentially make the manuscript of interest to the broad readership of The EMBO Journal.
Thank you for contacting me regarding the recent decision on your manuscript. I am in principle happy to consider a revised and rewritten manuscript, as I understand your concerns that the judgement of reviewer #1 could be affected by the presentation of the data. However, please note that I would send the revised manuscript back to the original reviewers, and since the final decision will be based on the reviewer comments on this version I cannot guarantee the outcome of this assessment.
Thank you so much for allowing us to revise the manuscript. We also wish to thank the referees for their excellent, professional comments, which tremendously assisted us in improving the manuscript. We comprehensively and thoroughly revised the manuscript according to their comments as follows.

**Referee #1**

1. 'Flexibility' is a bit of a misnomer since all polymeric complexes exhibit flexibility of varying degrees.
   We use the term ‘flexible’ for the mechanism of switching, not for the complex. We explain this both in the abstract and in the discussion (in the section “Unique features of the switching mechanism”). To avoid misunderstanding, we further clarified this point in these locations of the revised manuscript.

2. Nonetheless, the data is extensive and some of the experiments are interesting. However, I found it challenging to make sense of the proceedings. Several of my reservations stemmed from the lack of clarity in the description of the results. The parts that I did enjoy reading appeared to provide incremental information; the impact wasn't obvious to me.
   Regretfully, even though we worked very hard to make the original manuscript clear in spite of being quite elaborated with lots of experimental data, apparently, we did not succeed enough in doing so, as only one of the three referees wrote that the paper is clearly written. With the help of the referees’ comments, which clarified to us the points that were difficult to follow, we thoroughly revised the manuscript and made enormous efforts to make it easily readable and clearer. Furthermore, for better readability, we reduced the load of the presented data to the minimum essential, and removed parts of the story that were tangential to the main point.

3. The term 'biphasic' and 'monophasic' have not been described. Presumably the authors didn’t consider these to be important. However, large sections of the results have been dedicated to these minor effects.
   In the revised manuscript we now describe these terms. I should add that we do not consider the effects as minor. Yet, even if they were, they should not be ignored, because important information can be derived from them.

4. The authors strain to establish the relevance of short-lived switching to motility but it was not very clear whether it really influenced directional persistence.
   We limited the conclusions made about directional persistence and thoroughly revised the relevant section. Furthermore, because the main theme of the manuscript is two types of
switching behaviors associated with two different CheY-binding sites at the switch, we considered the issue of directional persistence as tangential to the main theme. Therefore, we shifted the revised section on directional persistence to the appendix (Appendix 2 in the revised manuscript).

5. A large part of the FRET responses observed in Fig 1B is likely due to the interactions between the thousands of cytoplasmic FliM and CheY-P molecules; they are in abundance compared to a few dozen FliM units within individual switches. Therefore, the FRET response is unlikely to be a good indicator of the nature of interactions between the mutant switch and CheY-P.

The referee is correct. We now realize that the data do not rule out the possibility that some of the FRET signal might have been due to interactions between CheY~P and free FliM molecules. In the revised manuscript we corrected the conclusions from the FRET experiments accordingly.

6. The appearance of an attractant response in the strain missing the kinase (negative control) is troubling. I wonder if the same complex phenomenon was responsible for the apparent weak attractant response in the tethered cell assay (Fig1D), in which case none of the experiments in Fig 1 really show an effect at the level of the flagellar switch.

Unlike the strong FRET responses to the attractant observed in kinase-containing strains, the response in the strain lacking the kinase was weak. This weak response might well be the reflection of the alternative pathway for chemotaxis, demonstrated in E. coli cells lacking most of the chemotaxis machinery but overexpressing CheY (Barak & Eisenbach, 1999, Mol. Microbiol. 31, 1125). We clarified this point in the revised manuscript. With respect to the “apparent weak attractant response in the tethered cell assay (Fig1D)” (Fig 2C in the revised manuscript), the response is slow (for the reasons mentioned in the text) but certainly not weak. As a matter of fact, the response to MeAsp is similar in magnitude to that of wild-type cells.

7. Further, overexpression of CheY is necessary to induce switching in the FliM\_\Delta N motors, and yet no repellent response was observed when treated with 1 mM Leucine (Fig 1E). This is very strange considering that the switch is ultrasensitive.

There is a misunderstanding here. First, we explicitly wrote in the discussion in the section “Gating can affect ultrasensitivity” of the original manuscript that “the ultrasensitive response of the motor was apparently lost when FliM\_N was removed (Figure 2A)” and we provided an explanation for the difference between a wild-type motor and a FliM\_\Delta N motor. In the revised manuscript we deal with this issue both in the results (first paragraph of the section “FliM\_\Delta N motors respond differently to CheY~P and CheY~Ac” and Fig 2G) and the discussion (the last paragraph of the section “Functions of FliM\_N”). Second, as mentioned in the legend to Fig 1 of the original manuscript (Fig 2C in the revised manuscript), the genotype of the strain used was \textit{fliM\_\Delta N \Delta cheZ}, meaning that CheY was fully phosphorylated. When CheY is fully phosphorylated, repellents, known to act by elevating the phosphorylation level, are not expected to work. All these are better explained in the revised manuscript (under “FliM\_\Delta N motors respond differently to CheY~P and CheY~Ac”).

8. A representative speed trace would have been more instructive here rather than the tethered cell depiction (Fig 1C).

In the revised manuscript we provide a representative speed trace, as suggested (Fig 2B).

9. I believe that a simpler interpretation of the results could be considered. Perhaps, the overexpression of CheY partially compensates for the lack of the N-terminus of FliM by
promoting nonspecific interactions within the switch. These non-specific interactions may not be relevant under wild-type expression levels of CheY.

We added a consideration of this possibility to the results of the revised manuscript (under “FliMΔN motors respond differently to CheY~P and CheY~Ac”). We mention there that the observation that CheY must be activated for generating clockwise rotation suggests that the clockwise generation was due to specific interactions of CheY with the switch rather than to nonspecific interactions within the switch, promoted by overexpressed CheY. We are aware of the possibility that nonspecific interactions might result from dispersion forces produced by the added charge of the phosphoryl moiety upon CheY phosphorylation. However, as we now indicate in the revised manuscript, this possibility does not hold here because CheY(D13K), which can be hardly phosphorylated, is as effective as CheY~P in generating clockwise rotation. Furthermore, as now mentioned in the results section “FliMΔN fusion to CheY compensates for FliMΔ truncation from the motor”, overexpression was not needed in the case of FliMΔN-CheY(D13K), where wild-type levels of this protein generated maximal clockwise rotation and, thus, completely compensated for the FliMΔN mutation (Fig 2H of the revised manuscript), implying that the interaction is specific.

10. It is not clearly explained by the authors what they meant by biphasic response in Fig 2B (page 5). I look at Fig 2B and I fail to see such a response. The authors follow up with the term 'biphasic trajectory' - what does that mean?

We thoroughly modified the text of the revised manuscript to clarify these points. In Fig 2B of the original manuscript (Fig 3A of the revised manuscript), two hyperbolic curves are observed, with only the first hyperbolic part emerges from the origin. This suggests two processes that differently depend on CheY concentration, i.e., biphasic dependence. A single process (monophasic response) would yield a single hyperbolic curve starting from the origin.

11. What do the authors mean by relaxation of the motor from CW to CCW; how could the kinetics of relaxation be possibly determined by the value of equilibrium constants as they claim on page 5?

Thanks for drawing our attention to the lack of clarity here. In the revised manuscript, we rewrote these sentences, avoiding the use of vague terms and clarifying the point to the best of our ability.

12. What is meant by monophasic response to acetate removal? At some point the authors decided to alter the meaning of bi- and monophasic responses from steady-state phenomena - Fig 2B purportedly demonstrates a biphasic response that depends on IPTG concentrations and not time - to kinetic phenomena (Fig 2D). This is difficult to follow especially since biphasic, monophasic, slow and fast phases appear throughout the manuscript and discussions.

Seeing the lack of clarity pointed to us by the referee, we thoroughly revised this section, trying to better explain our observations of biphasic kinetics and biphasic concentration dependence. In the discussion we also propose how these two biphasic phenomena are related to each other.

13. The authors have tried to establish some degree of relevance to these obscure effects by analyzing directional persistence (page 12). The section is unconvincing since 'angular shift' a key attribute has been defined as 'not tumbling behavior' - this could mean anything including loss of motility. The term is employed only once and has probably been superseded by 'angular deflection', 'turning angles' and so on in later instances.
We are sorry for the misunderstanding caused by the vague text here and by the variety of terms used for the same matter. Of course, we did not mean to define ‘angular shift” as ‘not tumbling behavior”. The definition is shown in Appendix Fig S9A and explained in Appendix 2 and in Appendix Supplementary Methods. Because the observations related to directional persistence are tangential to the main story, we shifted this section to Appendix 2 after revising it according to the comments of the referees.

14. Like several other places in the text, the description in this section is scientifically imprecise. The authors claim that they subtracted 'FliM_N-CheY measurements from the delta cheY measurements' - I don’t understand how measurements can be subtracted from each other - and then they find that the subtracted value peaks at 20 C (Fig 7C). Assuming that the probabilities are being discussed, it is unclear how meaningful, positive probability values were obtained by subtracting higher values (FliM_N-CheY) from lower values (delta CheY).

Again, bad wording. We subtracted the values of the \( \Delta \text{cheY} \) measurements from the values of the FliM_N-CheY measurements. Sorry. In Appendix 2 of the revised manuscript we now use a different analysis, proposed by referee 2.

15. Overall, large parts of the manuscript are unwieldy and impenetrable; some of the claims that I could track appear questionable at best.

We rewrote large parts of the manuscript to make it clearer. As mentioned above, we also reduced the load of the presented data by removing parts of the story that were peripheral to the main point.

Referee #2

1. The manuscript supplied a large amount of data on behavior of the bacterial flagellar switch in a FliM\_\Delta N strain, which led to interesting possible mechanism for motor switching, especially for the function of the two weak binding sites (FliN and FliM_M) of CheY-P. However, many places in the manuscript need clarification, some conclusions in the manuscript are too strong or incorrect, and many conclusions are inconsistent with one another. Please see below for comments.

As mentioned in the reply to referee #1, even though we worked very hard to make the original manuscript clear in spite of being quite elaborated with lots of experimental data, we regret that, apparently, we did not succeed enough in doing so. We now did an additional, immense effort to make the revised manuscript clear and easier to follow.

2. In the last paragraph of result 1: Different strains were used in Fig. 1E & F, please clarify the difference in the main text.

We now explain in the text both strains. Note that, as a result of the extensive revision, original Fig. 1F (Appendix Fig S4 in the revised manuscript) is now mentioned in the main text in another context (as a control of FliM\_\Delta N cells with intact chemotactic machinery).

3. In the last sentence: "These results suggest ... that acetylation is apparently more potent than phosphorylation for this." This conclusion is incorrect, as there is barely any change in CheY-P level in Fig. 1E for a cheZ-deletion strain with repellent stimulation, whereas the change in CheY-Ac level is significant in Fig. 1F.

Right. The conclusion was omitted from the revised manuscript.

4. In Fig. 2A, the binding affinity of CheY-P and CheY(D13K) to the motor are significantly different, please comment on the reason.
As we now mention in the text of the revised manuscript, the reason for the observation that the D13K mutation activates CheY better than does phosphorylation is not known. The D13K mutation is somewhat of a mystery; to our best knowledge, there is no complete and convincing explanation for why a CheY mutant, which is hardly phosphorylated, can show activation that surpasses the activity of phosphorylated CheY.

5. Near the end of Page 5: "This difference between the responses of FliM_N-CheY- and CheY expressing strains to acetate removal is consistent with the function of FliM_N as CheY activator, affecting the binding stability of the latter to the motor." Apparently from Fig. 2A the binding affinities for FliM_N-CheY and CheY to the motor are the same, please clarify the inconsistency.

There must be a mistake here because Fig 2A in the original manuscript (Fig 2G,H in the revised manuscript), which showed the dependence of clockwise rotation on the levels of CheY and FliM_N-CheY, certainly did not imply the same binding affinities of these two entities. The figure clearly showed that the binding of FliM_N-CheY (yellow curve in original Fig 2A; purple in revised Fig 2H) is much more intense than that of CheY (purple in the original Fig 2A and in revised Fig 2G). If, however, the referee meant to compare FliM_N-CheY~P (red) with CheY~P (cyan) (blue curves in revised Fig 2G,H), then he/she is right, because the affinities of these entities for the switch are similar, the only difference being the extent of binding (i.e., the level of saturation). In the revised manuscript we mention this point and conclude that the difference between these entities is not in the binding intensity but, likely, in the probability to be found in a conformation that is potent for clockwise generation. We further propose in the Discussion that CheY binding to FliM_N may stabilize a set of segmental conformations that is most adequate to act on the motor.

6. In Fig. S4, why do benzoate and leucine induce an attractant-like response, whereas in Fig 2C benzoate generate no response? Moreover, why the acetate-removal response in Fig. S4 (only slow decay) is different from those in Fig. 2D (fast decay or fast + slow decay)? How fast was the rate of addition or removal of the stimuli?

The difference between Fig S4 and Fig 2C in the original manuscript is that there is no kinase in the latter. Therefore, no phosphorylation-dependent response can occur in Fig 2C. The same goes for the difference between Figs S4 and 2D. The acetate-removal response in Fig 2D is solely a response to deacetylation whereas in Fig S4 it is mixed with a response to repellent withdrawal. In any event, as part of our efforts to make the paper clearer and more focused, we omitted Fig S4 and the related text from the revised manuscript.

With respect to the question about the rate of stimulus addition and removal, we now added to Methods that the time needed for replacement of the total chamber's volume was 6 s. Obviously, a response is expected to occur much sooner, i.e., as soon as the stimulus concentration starts changing in the chamber.

7. Near the end of the result section "CheY binding to the motor is biphasic": "we only measured spots of high intensity FliMΔN-YPet signal." Judging from Fig. 2B, it was impossible to measure motor spots. Since most of the fluorescence came from cytoplasm, it would be more convincing to look at signals form single motor instead of single cell. A TIRF measurement may be necessary.

Believing that the referee meant Fig 3B rather than 2B in the original manuscript, indeed it seemed impossible to measure motor spots from that figure. In the revised manuscript we replaced this figure with a higher-resolution figure (Appendix Fig S3B), which demonstrated spots of fluorescence. Nevertheless, for the reasons mentioned below, we are not certain anymore that we were able to measure CheY binding to the motor only. Our new conclusions from these experiments in the revised manuscript, now being included in
Appendix 1, reflect this change of interpretation. Thus, after reading the referee’s comment, we measured the FRET shift in the cytoplasm and in motors of a small dataset of single cells that were bleached one at a time. We consider this dataset to be of high quality because multiple timepoints were taken before and after photobleaching, and because the labeling of the motors was manually done. The examination of this dataset led us to two conclusions. First, the more CheY-mCherry was expressed, the more diffusive (i.e., hard to localize) the FliM\textsubscript{AN}-YPet spots became. And second, although the YPet signal in the cytoplasm was much lower than that at the spots, the FRET efficiency in the cytoplasm was equivalent to the FRET efficiency in the spots. Therefore, we shifted the description of this experiment to Appendix 1, where we only state conclusions that we can support, and we acknowledge the possible pitfalls of the measurement.

With respect to the suggestion to carry out a TIRF experiment: In our hands, TIRF measurements, carried out by us for other purposes, seemed insufficiently accurate and varied much between experiments. This insufficient accuracy (in our hands) combined with the fact that the photobleaching results are not essential for the conclusion of CheY binding to FliM\textsubscript{AN} motors (see revised manuscript), it seemed to us that investing more efforts in additional binding experiments in this rather restricted period is not necessary.

8. Also near the end of the result section "CheY binding to the motor is biphasic": "This is because clockwise rotation could be observed at CheY concentrations lower than those of the cooperative phase". This is inconsistent with what was claimed in result 1, where 100 \textmu{}M CheY was needed in a cheZ-deletion strain with FliM\textsubscript{AN} to observe CW rotation, which was larger than the K\textsubscript{d} of the cooperative phase. And probably more CheY-mCherry is needed due to the possible interference of mCherry. Moreover, the K\textsubscript{d} for the non-cooperative phase was about 11 \textmu{}M, which is similar to the value of 15 \textmu{}M for CheY-mCherry binding to FliM\textsubscript{wt} as said in result 1. So how the conclusion was reached that the non-cooperative phase was due to binding to FliN (a weak binding site)?

Thanks for calling our attention to this point. Due to the modifications made in the revised manuscript for the reasons mentioned just above (point 7), this stuff is no longer part of the manuscript.

9. In Fig. 4A, at low or high CW bias, the survival probability was claimed to be a single exponential. Then in Fig. 4B, how were the data at low CW bias for the second phase and the data at high CW bias for the first phase obtained?

Right. The wording in the original manuscript was, regretfully, misleading. We revised this section for both accuracy and clarity.

10. In Fig. 4D & Fig. S5, the peak reversal frequency (even with FliM\textsubscript{N} fused to CheY) is about 8-fold higher than the wild-type cells measured in ref. 34. And the biphasic decay for the survival probability was different from that observed in previous studies. However, the reversal frequency and the survival probability are sensitive to the analysis algorithm. A control experiment using a wild-type strain should be performed using the same data-analysis procedure, instead of just quoting the value from ref. 34.

Correct. We removed from the revised manuscript the comparison with wild-type cells. We would like to emphasize that the parameters and filters employed for our analysis were derived from a calibration experiment with a \textDelta{}cheY strain. Therefore, the measured clockwise interval lengths and reversal frequencies reflected true motor behavior.

11. In Fig. 4G, the FliN(A93D) mutant that impaired CheY binding still exhibited biphasic decay. This was inconsistent with the claim that the fast decay was induced by CheY binding to FliN.
Thanks for calling our attention to this apparent inconsistency. We incorrectly compared between FliN\textsubscript{wt} and FliN(A93D) cells containing different CheY variants. The FliN\textsubscript{wt} cells contained FliM\textsubscript{N}-CheY\textsubscript{~Ac}, whereas the FliN(A93D) cells contained FliM\textsubscript{N}-CheY\textsubscript{~P~Ac}. In the revised manuscript we made the correct comparison, with both strains containing FliM\textsubscript{N}-CheY\textsubscript{~Ac}. With the correct comparison, there was no inconsistency.

12. If the claim was correct that the fast phase in CW interval distribution was due to binding to FliN, and the slow phase was due to binding to FliM\textsubscript{M}, the lengths of those intervals were determined by the off rates for these two binding sites. If the FliM(E214W) mutation increased the affinity of FliM\textsubscript{M} for CheY as claimed, probably by decreasing the off rate, the slow phase should be even slower so that the separation between the fast & slow phase should be more obvious. This is in contradiction to what was observed in Fig. 5I.

This argument was right had the binding of FliM\textsubscript{N}-CheY\textsubscript{~P} to FliM\textsubscript{M} been an independent process. However, as our model proposes, the switching mechanism appears to be a gating mechanism, in which CheY binding to FliM\textsubscript{M} is dependent on the preceding binding to FliN. In the mutant, it is expected that each binding event to FliN would immediately proceed to FliM\textsubscript{M} binding due to the higher affinity of the latter for CheY. Indeed, the E214W mutant exhibited longer clockwise intervals, probably reflecting the stronger CheY binding to FliM\textsubscript{M}. This issue is better clarified in the revised manuscript (last paragraph of the Results).

13. On page 11 line 1-2: The conclusion was too strong. The extension of CheY dwell time at the switch by acetylation does not necessary mean FliM\textsubscript{M} has a preference for CheY-Ac. It only means that acetylation increases the CheY affinity.

Right. The text was modified accordingly.

14. The dwell time distributions (Fig. 6B-E) were obtained with wild-type motors. The effects of phosphorylation and acetylation on the two decay rates did not "suggest that the first and second clockwise phases are due to CheY interaction with FliN and FliM\textsubscript{M}, respectively, and that, apparently, CheY-P and CheY-Ac preferentially bind to the former and latter, respectively." (page 11, 3rd paragraph). As the authors suggested in the introduction, CheY binding to FliM\textsubscript{N} dominates over FliN and FliM\textsubscript{M} with the latter two being weak binding sites.

Correct. In the thoroughly revised manuscript, the conclusion from this experiment is minimal, saying that two distinct dwell times are indicative of two CheY-binding modes at the switch, either binding to two different binding sites at the switch or binding to two different states of the same site.

15. The claim above was also inconsistent with Fig. 3D and the conclusion at the end of the first paragraph on page 6, where CheY-P binding to the switch showed two phases, presumably one is binding to FliN and the other to FliM\textsubscript{M}.

As stated in the preceding point, the conclusion from that experiment was minimized and it now does not contain the claim.

16. Fig. 3D and Fig. 6H are inconsistent, with Fig. 3D showing two phases and Fig. 6H showing single phase. And the two phases in Fig. 3D were obtained with CheY-P, without involving CheY-Ac.

In view of the uncertainty related to the conclusions that can be drawn from the photobleaching FRET experiments (point 7 above), we removed from the revised manuscript the analysis shown in Fig 3 of the original manuscript.
17. The distribution of turning angles for swimming cells (Fig. 7B) depends sensitively on how the turning points were determined, especially when there were large amounts of small turning angles. Please provide details on how the turning points were determined.

The referee is right that the turning angle is very sensitive to how the turning points were determined. Therefore, we carefully made the determination, and devoted a section in Supplementary Methods of the original manuscript to the description of this determination. Noting that the original title of this section (“Automated analysis of cells swimming”) might have been misleading, we changed it in the Appendix Supplementary Methods of the revised manuscript to “Determination of the angular shift of swimming cells”.

18. The meaning of subtraction of two survival probabilities (Fig. 7C) is not straightforward, as the subtraction will be zero at 0 deg and large angles with a peak in between. I would suggest using the probability density distributions.

We revised the analysis as suggested and now use probability density instead of survival distributions (Appendix 2 and Appendix Fig S9 in the revised manuscript).

19. Last paragraph on page 13: "The observation that CheY binding to the switch is non-cooperative6,7 ...". More recent measurement (Fukuoka et al, Science signaling 2014, 7:ra32) actually demonstrated with single motor fluorescence that the binding is cooperative, which agrees with that modeled by Duke et al (JMB 2001, 308:541, Fig. 6). Please cite and comment on this.

Thanks for calling our attention to this omission. The studies of Fukuoka et al. and Duke et al. are now mentioned, in proper contexts, in the introduction, results and discussion sections of the revised manuscript.

20. The kinetic model of the switch is confusing (Page 14). Please provide more details. I do not understand the purpose of the model. Was it to explain the ultrasensitivity of the response of the wild-type motor (ref. 5)? Non-cooperative in the response of a FliM\_\DeltaN stain (Fig. 2A)? Or the ultrasensitivity of CheY-P binding? But the authors already claimed that the binding is non-cooperative by citing ref 6&7. I think Fig. 3D is probably worth to be explained by a model. Moreover, why assuming a single FliN-binding site and two FliM\_M-binding sites?

Thanks to this comment we realized that the kinetic model was, indeed, insufficiently clear and it did not provide significant information to the story. For this reason, the model was excluded from the revised manuscript. We provide in the Appendix another quantitative model (Appendix Fig S5) to explain the response of FliM\_\DeltaN motors to acetate removal (Fig 3B in the revised manuscript). As mentioned above, Fig 3D of the original manuscript is not included in the revised manuscript.

Referee #3

Overall, the paper is reasonably well organized and clearly written. The methods seem reliable, and the results and conclusion are sound scientifically. This research article would be of great interest to general readership, providing important advancements in our knowledge on directional switching mechanism of the flagellar motor. Although this reviewer thinks that the current form would be suitable for publication in EMBO Journal, this reviewer has only minor comments.

1. Potassium benzoate does not cause CheY acetylation but addition of 10 mM potassium benzoate at external pH 6.5 and pH 6.0 not only increase the switching frequency of the flagellar motor but also causes very long CW intervals, leading to tumbling of Salmonella cells (Sakai et al. mBio 10: e00079-19, 2019). This raises the possibility that the phosphorylated form of CheY can bind to FliMm in a way similar to CheY-Ac when the
concentration of CheY-P is high enough even in the absence of CheY-Ac. Therefore, it would be interesting to see whether 10 mM benzoate at external pH 6.5 and 6.0 causes biphasic CheY-binding to the motor in the absence of CheY-Ac.

Correct. At lower pH values, more benzoate enters the cell and, therefore, more CheY is expected to become phosphorylated. If CheY~P, when its level is high, can indeed bind to FliM, two phases should be seen also in response to benzoate at low pH. While this proposed experiment is a nice idea, we did not follow this advice for two reasons. One, practical, is that the lab of the corresponding author was closed due to retirement, and there is no one who can carry out the experiment. Furthermore, the COVID-19 circumstances prevent the first author from examining this question in his current host lab. The other is the readability of the manuscript. The referees’ comments above, primarily those of referee 1, indicated that the manuscript was hard to follow. Therefore, we reduced, for better readability, the load of the presented data to the minimum essential, and removed parts of the story that were tangential to the main point. We feel that doing the opposite and adding a nice little experiment, which is not essential for the main story, would be counterproductive in this respect.

2. The authors should cite the following paper because this paper is the first report describing the dissociation rate of CheY from the rotating motor in single E. coli cells. Fukuoka H, Sagawa T, Inoue Y, Takahashi H, Ishijima A. Direct imaging of intracellular signaling components that regulate bacterial chemotaxis. Sci Signal 7: ra32.

Right. Thanks for calling our attention to this omission. The study of Fukuoka et al. is now mentioned, in proper contexts, in the introduction, results and discussion sections of the revised manuscript.
Thank you for submitting a revised version of your manuscript. The study has now been seen by two of the original referees. Reviewer #2 was unfortunately not able to review the revised version. While both reviewers appreciate the added information, reviewer #3 also indicates several aspects that would have to be clarified in the final revised manuscript before I can extend acceptance of the manuscript. Therefore, I would like to invite you to address the remaining referee comments and the following editorial issues.

****************************************************************

- Referee #1:
- I reviewed an earlier version of this work.
- Strengths:
  1. Thanks to the improved writing in the present version, I am able to appreciate the key results and the motivation better. Other works showed that a phosphorylated regulator, CheY, interacts with two types of proteins in the flagellar motor, FliM (specifically FliM_N) and FliN to activate the motor switch. In the present work, the authors show that when acetylated, CheY can bind to FliM_M to stabilize CW rotation. This is finding will interest readers in the field.
  2. That the FliM_M has low affinity to CheY is not new; it has been discovered previously in a different species. But, I do not think this is an issue as the authors have extensively characterized the effects of these interactions on the response of the switch in a E. coli. The authors have also appropriately referenced the literature.
  3. Experimental characterization appears to be rigorous and performed with great care. The extensive results support many of their conclusions. This referee particularly loved the idea of fusing FliM_N with CheY to study its role - nice experiment!

Weaknesses:

1. In the experiments where CheY binding to FliN is impaired, the authors suggest that "Short clockwise intervals were hardly detected, suggesting that CheY binding to FliN apparently generates short intervals of clockwise rotation". This appears incorrect as the relevant plots show a very high probability of observing short intervals (Fig 4B). The plot indicates that there is a high probability of observing long intervals when CheY-FliN interaction is impaired, which could merely be a consequence of a higher CW level (I can't tell the CW level; the colors look all similar). Are the authors confusing slopes in probability vs. intervals with actual probabilities? If yes, then it appears as though the data is more consistent with a conclusion that CheY binding to FliN inhibits long CW intervals. Subtle but important difference as FliN - CheY interactions do not appear to be necessary to observe short intervals. In that case, go/no-go and other ideas appear questionable.

2. That there would be some long-lived and sometimes short-lived intervals in a random process such as motor-switching goes without saying. A great emphasis is placed on short vs. long intervals without mentioning that this is but a natural property of stochastic processes.
3. The monophasic/biphasic description could be made clear by focusing on the decay following acetate removal - that would clearly show what the authors mean with biphasic and monophasic responses - at present this is not clear, especially the latter (Fig 3B). Also, the authors don't seem to need or dwell on Fig 3A much (unless I missed some discussion related to it). This sub plot can be moved to the supplementary to help improve readability; otherwise the issue about biphasic saturation versus biphasic kinetic response will likely be a source of confusion.

4. I greatly appreciate the authors' efforts to address the previous issues with writing. But, the results in current form are likely to engage only the expert reader. The authors could improve its broader appeal by borrowing from the clarity in the Discussions to the results and elsewhere, especially the abstract. Another suggestion would be to keep sentences short where possible, and limiting unrelated information. Example - "Independently of whether such two phases only occur in FliMΔN motors or whether they also occur in FliMwt motors but masked there by the stronger binding to FliMN, the findings of two phases suggest that at least two processes are involved in motor switching." Minor errors - end of page 3 reads 'i.e, for the probably to'. Also 'vigor turning motion' to describe a tumble is unusual (introduction).

5. In the abstract, the authors say the motor is exceptionally ultrasensitive (to what?). They claim that they have identified in this work "three binding sites and two different covalent modifications", which gives the false impression that all of these binding sites and modifications have been newly discovered here. More importantly, binding interactions with what? For a reader unfamiliar with the problem, covalent modifications and strict binding sequences mean very little - CheY hasn't been mentioned.

6. That the newly discovered interactions are likely in play only when CheY is acetylated dampens enthusiasm somewhat.

-Referee #3:

The revised manuscript has been significantly improved based on the comments and suggestions raised by the three reviewers. This reviewer wanted the authors to perform additional experiments to clarify their model but understood a reason why they did not.

Therefore, this reviewer did not have any additional concerns and felt it would be acceptable for publication in EMBO Journal.
2nd Authors' Response to Reviewers

The authors performed the requested editorial changes.

Referee #1 - Weaknesses

1. In the experiments where CheY binding to FliN is impaired, the authors suggest that "Short clockwise intervals were hardly detected, suggesting that CheY binding to FliN apparently generates short intervals of clockwise rotation". This appears incorrect as the relevant plots show a very high probability of observing short intervals (Fig 4B). The plot indicates that there is a high probability of observing long intervals when CheY-FliN interaction is impaired, which could merely be a consequence of a higher CW level (I can't tell the CW level; the colors look all similar). Are the authors confusing slopes in probability vs. intervals with actual probabilities? If yes, then it appears as though the data is more consistent with a conclusion that CheY binding to FliN inhibits long CW intervals. Subtle but important difference as FliN - CheY interactions do not appear to be necessary to observe short intervals. In that case, go/no-go and other ideas appear questionable.

The referee is correct about the sentence "Short clockwise intervals were hardly detected, suggesting that CheY binding to FliN apparently generates short intervals of clockwise rotation". The sentence is corrected in the revised version. We now explain that the slope of the decay (Fig 4B) was similar to the decay slope of long clockwise intervals in FliN_{wt} motors (Fig 3D), suggesting that CheY binding to FliN apparently generates short intervals of clockwise rotation. We also now indicate that this conclusion is consistent with the much lower reversal frequency of FliN(A93D) motors than that of FliN_{wt} motors (Fig EV2B,C versus Fig EV1A,C, respectively). Furthermore, the referee raises a concern that the high probability of long intervals when CheY-FliN interaction is impaired could merely be a consequence of a higher CW level. This possibility does not hold because, as shown in the figure, the clockwise levels are the same in wild-type cells and FliN-impaired cells in each color code.

2. That there would be some long-lived and sometimes short-lived intervals in a random process such as motor-switching goes without saying. A great emphasis is placed on short vs. long intervals without mentioning that this is but a natural property of stochastic processes.

We added to the revised manuscript this point.

3. The monophasic/biphasic description could be made clear by focusing on the decay following acetate removal - that would clearly show what the authors mean with biphasic and monophasic responses - at present this is not clear, especially the latter (Fig 3B). Also, the authors don't seem to need or dwell on Fig 3A much (unless I missed some discussion related to it). This sub plot can be moved to the supplementary to help improve readability; otherwise the issue about biphasic saturation versus biphasic kinetic response will likely be a source of confusion.
We rephrased some of the text to make it clearer. With respect to Fig 3A: Since the occurrence of two phases in several related processes is a central thread of this study, the relevant data should be easily accessible to the reader. We, therefore, think that inclusion of the biphasic clockwise dependence in Fig 3A is important for the benefit of readability and understanding of the paper.

4. I greatly appreciate the authors' efforts to address the previous issues with writing. But, the results in current form are likely to engage only the expert reader. The authors could improve its broader appeal by borrowing from the clarity in the Discussions to the results and elsewhere, especially the abstract. Another suggestion would be to keep sentences short where possible, and limiting unrelated information. Example - "Independently of whether such two phases only occur in FliMΔN motors or whether they also occur in FliMwt motors but masked there by the stronger binding to FliMN, the findings of two phases suggest that at least two processes are involved in motor switching." Minor errors - end of page 3 reads 'i.e, for the probably to'. Also 'vigor turning motion' to describe a tumble is unusual (introduction).

As requested, in the revised manuscript we wrote the abstract more clearly, we avoided too large sentences throughout the manuscript, and we corrected the pointed minor errors.

5. In the abstract, the authors say the motor is exceptionally ultrasensitive (to what?). They claim that they have identified in this work "three binding sites and two different covalent modifications", which gives the false impression that all of these binding sites and modifications have been newly discovered here. More importantly, binding interactions with what? For a reader unfamiliar with the problem, covalent modifications and strict binding sequences mean very little - CheY hasn't been mentioned.

We rephrased the relevant text to address these points.

6. That the newly discovered interactions are likely in play only when CheY is acetylated dampens enthusiasm somewhat.

We added to the Discussion (Under “The switching mechanism: an apparent gating mechanism”) a referenced reminder that CheY~Ac is always present in the cell, meaning that there is no reason for dampening the enthusiasm.
Thank you for addressing the remaining issues in the revised manuscript. I sincerely apologise for the delay in processing of your manuscript due to the holiday period and the unusually high manuscript submission rate to our office at the moment. I am now pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.
In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

**B- Statistics and general methods**

| Question                                                                 | Answer |
|--------------------------------------------------------------------------|--------|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | NA     |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA     |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | None   |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment? (e.g., randomization procedure?) If yes, please describe. | Yes. The analysis was automated, where possible. Thus, all samples were treated by the same unbiased script. |
| For animal studies, include a statement about randomization even if no randomization was used. | NA     |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or, and when assessing results (e.g., blinding of the investigator)? If yes please describe. | Some experiments in the same dataset were replicated by the lab technician while the investigator was blind to the workflow. All observations, including ones that were made by the lab technician, were blindly automatically analyzed by the same automatic workflow, where it was possible. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA     |
| 5. For every figure, are statistical tests justified as appropriate? | There is only one statistical test in the appendix, and it is a non-parametric test. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | The statistical test we employed in appendix 2, which is the only statistical test in the manuscript, is a non-parametric test. |
| In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable). | Yes, though in many cases we only provide a reference to the raw data in the appendix to not clutter the figure with estimates of variation. |
| C - Reagents |   |
|--------------|---|
| 8. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia [see link list at top right], Biolegend [see link list at top right]. | N/A |
| 9. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | N/A |

| D - Animal Models |   |
|-------------------|---|
| 10. We recommend consulting the ARRIVE guidelines [see link list at top right] (Fizelle Biol. R[3] 1; 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. See also: NIH [see link list at top right] and MRC [see link list at top right] recommendations. Please confirm compliance. | N/A |

| E - Human Subjects |   |
|-------------------|---|
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines [see link list at top right]. Please confirm you have followed these guidelines. | N/A |

| F - Data Accessibility |   |
|------------------------|---|
| 16. Provide a “Data Availability” section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE13462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for ‘Data Deposition’. | Links are provided for the data. |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in a Supplementary Document (see author guidelines under ‘Expanded View’ or in unstructured repositories such as DDBJ (see link list at top right) or Figshare (see link list at top right). | Links are provided for the data. |

| G - Dual use research of concern |   |
|-------------------------------|---|
| 22. Could your study fall under dual use research restrictions? | No |