In the present manuscripts the authors aim at characterising the chaperone trigger factor (TF) regarding the dimerisation of TF and its implication on interaction with substrates. If the topic is interesting and trying to answer biologically relevant questions, the overall quality of the paper appears poor and too preliminary for publication.

1. There is a lengthy discussion about TF assignments. As presented here, RDB and PPI domains were assigned previously by the authors, SBD and TF113-432 by Dyson's group and the full length by Kalodimos and Hiller groups. It thus seems to me that all this constructs were assigned before. Please clarify this section and state explicitly your contribution to the field.

   As per the reviewer's suggestion, we have substantially shortened the literature review of the previous NMR work, but still acknowledge their contributions that enable us to carry out the NMR PRE analyses as described in this work.

2. For characterising the dimer interface and dynamics, the authors use a combination of NMR line-widths analysis and EPR. This is an interesting approach, however the obtained data remain low resolution and do not allow for a clear description of the dimer properties. This section is thus not very conclusive and as the authors have all the tools in hand to label with MTSL each domain of TF, I am wondering why they do not prepare mixture of isotopically labelled TF with each of the MTSL constructs successively to answer which domains of TF are in fact interacting or in close proximity. Extra EPR measurements could be used to derive informations about the domain even if more distant than the PRE distance range.

   We appreciated the reviewer's suggestion, and have now included the DEER measurements that indeed provide additional structural information that is not accessible by NMR-based analysis. The ESR-based DEER results showed that the previously reported TF dimer structures reported by Kalodimos and Hiller's groups sample part of the conformational space that is probed by ESR. The description of the DEER analysis is included in the revised manuscript (lines 196-215), as follows:

   The severely broadened methyl proton resonances of the RBD residues and faster T_2 relaxation of the spin label at 14C likely correspond to the conformational heterogeneity within the dimer interface. Indeed, a number of different TF dimer structures have been reported by two independent studies based on different NMR restrains (Morgado *et al.,* 2017, Saio *et al*., 2018). To investigate the TF dimer conformations through ESR spectroscopy, we carried out double electron-electron resonance (DEER) measurements to determine the inter-spin distance distributions of different combinations of spin-labeled TF samples. These included the uniformly single species or the 1:1 mixture of two variants (denoted as site A'/site B). Figure 2 shows the distance distributions extracted from the DEER time-domain data (Supplement Fig. S2) using the Tikhonov-based regulation methods (Lai *et al*., 2019; Chiang *et al*., 2005a). The DEER distance distributions (solid lines in Fig. 2) are compared with the predicted inter-spin distance distribution (shaded areas in Fig. 2) calculated from the three previously reported NMR structures (Morgado *et al.,* 2017; Saio *et al*., 2018) using the MtsslWizard program (Hagelueken *et al.*, 2015). In general, the DEER distance distributions show multiple distinct populations indicating conformational heterogeneity in the TF-dimer. While the majority of the DEER-derived peak distributions could find correspondences from the NMR structures, a few discrepancies did exist. They were indicated by asterisks in Fig. 2. Specifically, the DEER measurements identified a shorter distance pair for 14'/14 centered at approximately 3 nm, when all reported NMR structures showed corresponding distances at 4 nm and above. Likewise, the DEER-derived distance distribution of 326'/326 showed an additional peak at approximately 4.7 nm, but it was not present in the NMR structures. Furthermore, the DEER-derived distance distributions of 14'/326 and 14'/376 showed three distinct populations, which were in agreement with the conclusion drawn by the CW-ESR analysis that the RBD exhibits abundant structural heterogeneity. Overall, the RBD (14C) exhibited a higher level of conformational heterogeneity than what was previously determined by NMR spectroscopy. Collectively, our ESR analyses clearly demonstrated the abundant structural polymorphism of the TF dimer in solution

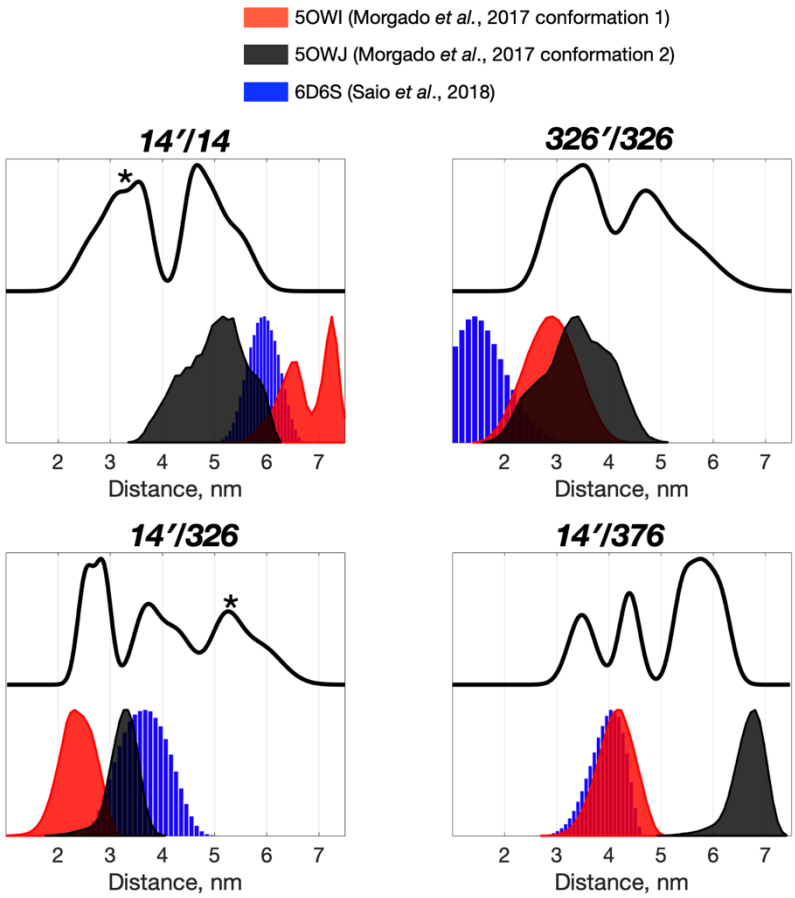

Figure 2. **Multiple dimeric TF conformations revealed from the DEER measurements**. DEER samples were prepared by either the single species or the 1:1 mixture (denoted as site A′/site B) of the three single-cysteine variants, 14C, 326C and 376C. DEER distance distributions of TF dimer (solid line) were compared with the distance distributions calculated from the previously determined TF dimer structure (PDB codes: 5OWI (red), 50WJ (black), and 6D6S (blue)). There are a few discrepancies between the DEER and NMR results, as indicated by asterisks.

3. In the section regarding interaction with peptides, the authors aims at verifying the quality of a theoretical model model regarding TF-target interactions. This is an interesting question however the current approach lack rigorous testing. In fact the selection of the 5 constructs remains unclear to me. For exemple why is the site around 290 not chosen? It seems from the figure to be a potentially better site than 5. Also no testing on a site which is predicted to not or poorly interact is done.

   The five peptide sequences were selected not only based on the predicted binding scores but also based on the previously reported peptide array data by Deuerling *et al.*, 2003. Visual inspection of the blotting intensities of individual peptide fragments showed that the site around residue 290 has little TF binding. So we did not choose this region for peptide synthesis. To clarify this issue, we included a statement in line 224 that reads:

   "As a model system, we correlated the previously reported peptide array data of TF binding to ICDH (Deuerling et al., 2003) and the predicated TF binding score as a function of ICDH sequence (Fig. 3a). By visual inspection of the blotting densities of the peptide array, we identified five segments within the ICDH sequence that showed strong TF binding and fulfilled the requirement of peptide length and composition (Table 1). We adjusted the window sizes of the selected sequences to maximize the amount of preferred amino acid types and chemically synthesized these peptides."

   We hope the reviewer will find this explanation acceptable.

4. In the comparison of the interaction between IcdH2 and IcdH3, in the actual format it is almost impossible to assess the quality and relevance of the data and analysis. The PRE figures are extremely hard to read. Please adjust those figures, possibly with a multiple panel organisation so that the data readable and easily comparable between the different considered systems/probes. Please also indicate where each domain is in the sequence. For exemple, when the authors indicate that "the loss of PRE was much more pronounced for IcdH3 compared to that of IcdH2", I couldn't find any quantitative comparison or direct comparison of the experimental data. The conclusion of this section regarding TF dimerisation seems to me quite speculative regarding the current data. It might be possible however to better reach those conclusions if the data where more adequately presented.

   We appreciate the reviewer's critic of our data presentation. The PRE data are replotted individually for the backbone amide and side-chain methyl groups in Figures 4 and 5.

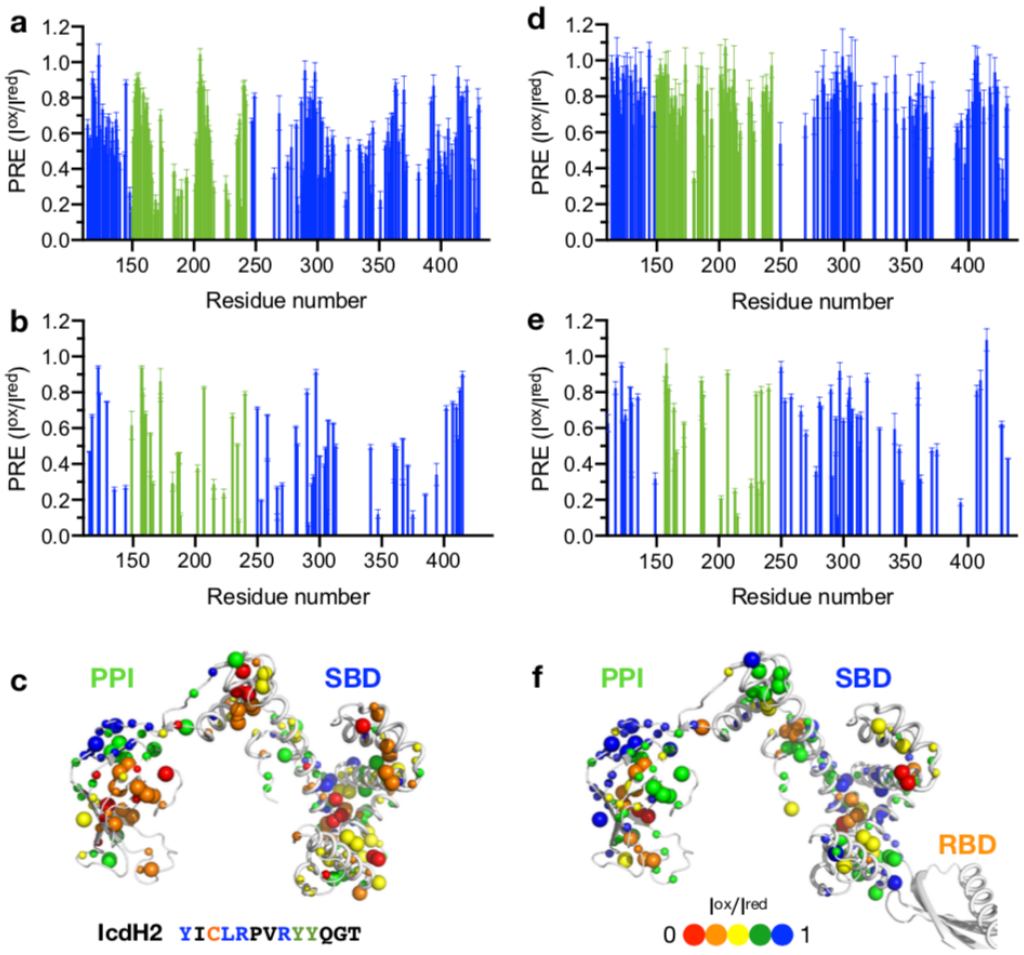

Figure 4. Structural mapping of the PREs induced by the MTSL-labeled IcdH2 peptide on TF without and with the RBD. The backbone amide-based PREs of PPI+SBD (a) and full-length TF (d). The side-chain methyl-based PREs of PPI+SBD (b) and full-length TF (e). Structural mapping of the observed PREs onto the structure of PPI+SBD (c) and full-length TF (f). The backbone amide nitrogen atoms and side-chain methyl carbon atoms are shown in small and large spheres, and are color-ramped from red to blue, corresponding to small and larger PREs as indicated by the filled circles below. The observed PREs expressed as the ratio of the peak intensities of the oxidized (paramagnetic state) over the reduced (diamagnetic state) states (I^{ox}/I^{red}) as a function of residue number between 113 and 432. The PRE values corresponding to PPI and SBD are colored in green and blue, respectively. The residues corresponding to the RBD are omitted due to the severe line broadening that precludes reliable data analysis.