Responses to RC3

To RC3:

Takeda, Kainosho and co-workers present an in-depth investigation of lysine residues in a variant of SNase that has been engineered to harbor a lysine residue in its core, of which the pKa constants i shifted by about 5 units to below physiological pH. They make elegant use of their SAIL technology for stereo-specific protiation/deuteriation of the side chain, and demonstrate several approaches for the investigation of side chain conformation and charge state. The work displays a high degreee of technical rigor and is well-documented. The paper is also examplary when it comes to scholarly presentation and quality of illustrations. At the same time it is unclear to what extent the presented methods are going to alter the ways in which Lys pKa constants are studied, and the suggestion that isotope shifts on the 13Cε might be used as a proxy for Lys charge state is not yet sufficiently substantiated. It is clear from published data, as well as from results in the report that the chemical shifts of various side chain 13C and 15N atoms emerge as very powerful reporters. This has previously convincingly been demonstrated by André et al (Andre et al. JACS 2007; https://doi.org/10.1021/ja0721824), and also by the group of R.E. London (Gao et al. JACS 2006; doi: 10.1021/ja061473u) and it would appear that these methods would remain those of choice? The current work does, however, clearly point a path forward. As the Kainosho group has clearly demonstrated, access to partially deuteriated side chains displays significant improvements for higher molecular weight proteins, where the methods mentioned before will probably fail. There, utilization of SAIL Lys can present significant advances. Gauging charge state of amino acids based on chemical shifts alone presents a small uncertainty, but it is unclear at this point that isotope shifts would prove more reliable. Ultimately,either (a) the method put forward by McIntosh and co-workers (Poon et al. JACS 2006; https://doi: 10.1021/ja065766z) where the multiplet pattern of the amino groups is observed, or (b) observing a titration in the 13C or 15N shifts in the HECENZ NMR experiment would remain the unambiguous approaches. Therefore, I think that the title might do better justice when "revealed" would be replaced by "studied" or "investigated". In similar vein, the abstract might be overly optimistic to state that the isotope shift "will" be a powerful tool - possibly it "might" (Indeed on p18, the authors use a more cautious formulation). Also the 13C 1D spectrum of ε-[13C,d2]-Lys is not as dispersed as one would wish, and for larger proteins than SNase may present severe shortcomings when compared with 2D NMR. It is unfortunate that the authors were not more successful with HECENZ experiments using SAIL-Lys (as judged from Table 1, where several 15Nζ shifts are missing).

Response: Thanks for the comments. According to the related comments by RC1 on the HECENZ experiment, we added the spectrum (Fig. A4) in the Appendices. As one can see from the spectrum

(Fig. A4a), we could actually determine the $^{15}N^{\zeta}$ chemical shifts for all of the 21 Lys residues in the SNase variant, although some of them are nearly identical. It should be noted that the $^{1}H^{\varepsilon 2}$-$^{15}N^{\zeta}$ correlation signals in the HECENZ spectrum for the residues that showed isolated signals in both the 1D $^{13}C^{\varepsilon}$ spectrum (Fig. 4a) and 2D $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ spectrum (Fig. A4b); i.e., K66, 78, 136, 133 and 9, are clearly separated from the congested spectral region. As stated in the revised abstract, those residues are likely to exist in an unusual local environment and might have peculiar ionization states. The deuterium-induced isotope shifts for $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ of these residues could be accurately estimated, as compared to the other residues. We didn't include the $^{15}N^{\zeta}$ chemical shifts for K5, 6, 63, 70 and 97 in the previous Table 1, since their sequential assignments were not firmly established due to the incidental signal overlapping of their $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ signals. The isotope shifts for the $^{15}N^{\zeta}$ data of these five residues were excluded in Table 2, since the uncertainties were larger than those for the other residues.

Although the manuscript is very clearly written, I have a few textual comments:

p5 l111 - when referring to "standard protocol", a reference should be given
Response: We cited the paper by Isom et al., describing the expression and purification protocol for SNase variants.

p5 l111 - labelling "rates"; do the authors refer to incorporation level?
Response: Yes, but we replaced "labeling rates" by "enrichment levels".

Methods section - the chemical shift referencing procedure is missing. Was it IUPAC (Markley et al) or Bruker?
Response: We described the chemical shift referencing procedure in section 2.1 and 2.2.

p6 l196 - "outrageous" probably means "outlier"? I urge the authors to be more transparent about how this was done
Response: According in BMRB deposition, "outliers" are defined as those with > 8 σ from the mean of the chemical distribution histograms. Therefore, we deleted this misleading expression and recalculated the averaged chemical shift values in Table 1 by excluding those for Lys-66.

p12 Could the ring currents that are discussed possibly be predicted from the structure, and utilized?
Response: The discussion about the aromatic ring current shifts for the Lys sidechain NMR signals is not quantitative. We just compared the high-field shifted signals to the crystal structure of the SNase variant. We are hoping to measure the NOEs between the Lys-chain and aromatic ring proton

signals using SNase simultaneously labeled with SAIL-Lys and SAIL-Tyr/Trp. With such *quantitative* data, the comparison between the aromatic ring current shifts predicted by the crystalline structure and the NMR data would be interesting.

p12 l257 - rather than being "quite useful" is would appear that 15Nζ shifts would be "decisive" or "unambiguous"?

Response: We fully agree with RC3 that the $^{15}N^{\zeta}$ shifts, if accurately measured and sequentially assigned, would be “decisive” information for the ionization states of the Lys ζ-amino groups. Nevertheless, the present method using $^{13}C^{\varepsilon}$ isotope shifts would still serve as a useful initial step to characterize the residues showing distinctive $^{13}C^{\varepsilon}$ signals for an [ε; ε,ε-D_2]-Lys labeled protein, which may have deprotonated Lys residues under certain physiological conditions, possibly due to an unusual local environment stabilizing the deprotonated ζ-amino group.