In a succinctly written paper, Takeda and co-workers present the use of selective stereo-array isotope labeling (SAIL) with [U-^{13}C,^{15}N; β2,γ2,δ2,ε3-D_4]-Lys to completely and stereospecifically assign the sidechain ^{1}H, ^{13}C and ^{15}N signals of lysine residues in proteins. In addition, along with the use of [ε-^{13}C;ε,ε-D_2]-Lys labeling, this approach elegantly facilitates the measurement of $^{13}C^{\varepsilon}$ isotope shifts accompanying deuteration of the lysine terminal amines (N^{ζ}). As exemplified with the Δ+PHS/V66K variant of *Staphylococcal* nuclease (SNase), the resulting chemical shifts and deuterium isotope shifts can provide insights into the structure and ionization states of lysines in a protein. Thus I certainly recommend that this interesting paper be accepted for publication in the special issue of *Magnetic Resonance* after consideration of the following hopefully helpful comments.

Specific comments:

• line 34 and the first introductory paragraph - Although the SAIL approach certainly facilitates the characterization of lysine residues, I do not agree that the information "could not be obtained by other methods" and that there only been a "only a few systematic NMR studies" on lysines. Indeed, an inspection of the BMBR (https://bmrb.io/ref_info/csstats.php) shows extensive chemical shift assignments for lysine ^{1}H, ^{13}C and ^{15}N nuclei. Also, a simple Web of Science search for "lysine and NMR" yields 4486 results.

• lines 74, 256 and 263 - It would be worth emphasizing (rather than unnecessarily downplaying) that the $^{15}N^{\zeta}$ and $^{13}C^{\delta}$ (but not the $^{13}C^{\varepsilon}$) actually serve as an excellent reporter nuclei for determining the ionization states and, when monitored as a function of pH, the pKa values of lysine residues in proteins (Gao et al. *J.A.C.S.* 128:8104; reviewed in Platzer et al. *J. Biomol. NMR* 60:109). This can be seen for Lys66 in Table 1.

• line 100 - Could the authors comment on the use of an *E. coli* lysine auxotroph (*lysA*) to improve labeling efficiency (Waugh *J. Biomol. NMR* 8:184)?

• Figure 1 - Given the importance of the $^{15}N^{\zeta}$ signals, it might be useful to also include the HECENZ spectrum of SNase in this figure, or as a supplemental figure.

• line 223 and elsewhere - Although often referred to as "hydrophobic interactions" (i.e., a non-specific exclusion from water), the specific packing of the lysine sidechains within SNase are better described as resulting from "van der Waals" interactions.

• Line 307 - The pH-dependent deuterium isotope shifts of lysines were investigated by Led and Petersen *J. Mag. Res*. 33:603 (see also Led et al. *J. Mag. Res*. 20:530, Tomlinson et al. *J.A.C.S*. 131:4674, and reviewed in Platzer et al. *J. Biomol. NMR* 60:109).

• abstract, section 3.3., and lines 411 to 413 - Certainly an interesting and most emphasized result from this study is that deuterium shifts of the lysine $^{13}C^{\varepsilon}$ signals appear to depend on the ionization states of the ζ-amino group (~ -0.3 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3$+-$N^{\zeta}H_3$+] versus ~ -0.2 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$]). Although consistent with the results reported by Led and Petersen *J. Mag. Res*. 33:603, it is not clear than such small isotope shifts can provide unambiguous evidence for the ionization states of lysines in proteins. The shifts were only measured at a single sample pH/pD value (where all lysines in SNase are positively charged, except for the sole Lys66 being neutral). To draw this conclusion, the isotope shifts should be monitored over the course of pH/pD titrations. Furthermore, the value of ~ -0.2 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] was derived from *only* Lys66, which has a very perturbed pKa value of 5.7 due to its burial within SNase. An unusual environment may well cause an unusual isotope shift. Indeed, for the protonated amines, the $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3$+-$N^{\zeta}H_3$+] values in Table 2 span from -0.39 ppm to -0.28 ppm, i.e. a range of 0.11 ppm. The causes for such a range might be worth commenting upon. However, if $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] spanned a similar range, then the deuterium isotope shifts for protonated versus neutral lysines would overlap and could not be used to unambiguously determine their ionization states. Accordingly, it would be very useful if the authors could measure the $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] values for SNase at pH/pD > 12.5. These could be extracted from two 1D spectra of [ε-^{13}C;ε,ε-D_2]-Lys labeled SNase as

assignments would not be necessary. The goal would be to determine the range of $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] values exhibited for neutral lysines in a protein. Admittedly, this could be challenging as the stability of SNase falls off rapidly at pH > 10 (Garcia-Moreno et al. *Biophys. Chem*. 64:211). Alternatively, numerous variants of SNase with buried lysines having pKa values in the accessible range of 5 - 9 could be investigated (Isom et al. *P.N.A.S*. 108:5260). Of course, this may be beyond the scope of this paper. Nevertheless, the authors should discuss the concerns outlined above when effectively proposing that small deuterium isotope shifts are more reliable and easily measured than very diagnostic $^{15}N^{\zeta}$ and $^{13}C^{\delta}$ chemical shifts for determining lysine charge states. In the end, these approaches (and others, such as measuring $^{15}N^{\zeta}$-$^{1}H^{\zeta}$ scalar couplings) provide complementary insights that collectively re-enforce a more complete view of the structural, dynamic and electrostatic properties of lysines in proteins.

Technical corrections:

• lines 29 and 89 - "contains as many as 21 Lys resides" could be replaced with "contains 21 Lys residues".

• line 66 - It would useful to provide references for cases where the "ionization states of Lys ζ-amino groups in a protein have been characterized by X-ray crystallography." Except with neutron or ultra-high resolution X-ray crystallography, the ionization states of lysines are generally inferred from physical arguments.

• lines 99 and 100 - It would help to include the reference (Terauchi et al., 2011) for the source of the SAIL-Lys, which presumably the authors prepared in-house (or is it commercially available?). Similarly, what were the sources of the [U-^{13}C,^{15}N]-Lys and [ε-^{13}C;ε,ε-D_2]-Lys? Also, were these pure L-enantiomers or DL-racemic mixtures?

• line 111 - "labeling rates" could be replaced by "enrichment levels".

• line 196 - "outrageous" could be replace by "outlying".

• line 258 - "Lys residues, given that ..." could be replaced by "Lys residues. However, the ..."

• line 391 - "exchange rate constant (k_{ex})", rather than "exchange rate (K_{ex})"

• line 393 - s^{-1}, rather than S^{-1}

• Table A1 - Dihedral angles should be rounded off to reflect realistic precision (i.e., not to 0.1°).