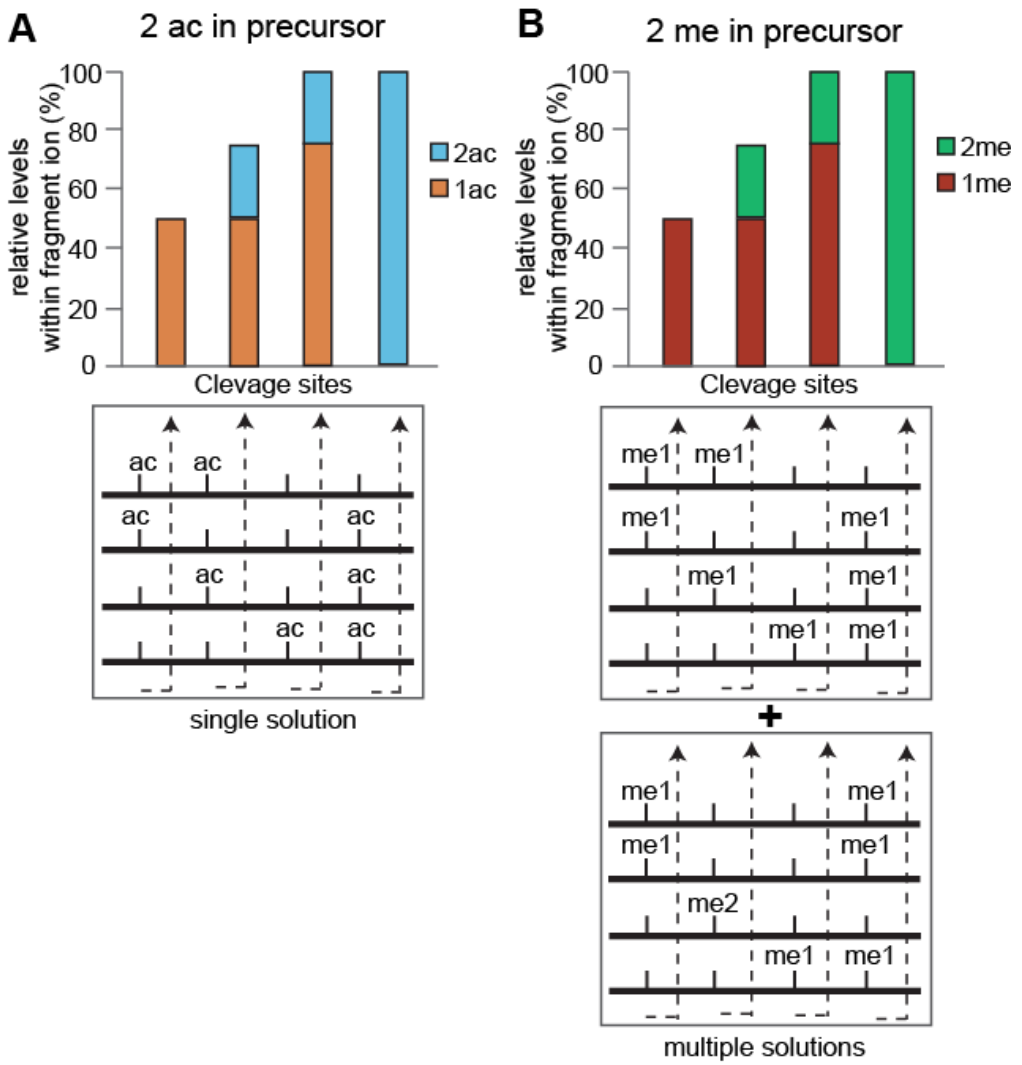

Fig S5. Convolving effects of methyl groups when assigning modifications using MS^2 intensity.

Analogous to different proteoforms with same precursor mass in Bottom-Up proteomics is positional isomers. One of the extensively studied positional isomers is acetylations of lysines 5, 8, 12, and 16 in histone H4 by us and others (1, 2). Mono-, di-, tri-, and tetra-acetylated forms have been observed from proteolytic peptide encompassing these four sites. It is possible to characterize these positional isomers by MS^2 experiments. However, quantitation of the relative levels of these isomers by solving multiple linear equations is possible for four mono- and tri-acetylated isomers but becomes impossible for six di-acetylated isomers [2]. This is because the number of linear equations afforded by MS^2 experiment is less than parameters necessary to deconvolve the relative levels for six positional isomers in di-

acetylated H4 (K5, 8, 12, and 16). Therefore, a recent study has resorted to MS^3 to quantitate six isomers of diacetylated H4 peptide spanning from residue 4 to 17 (3). The complexity of positional isomers generated by methylation is even greater due to three different methylation states as mentioned in **Table S2** and the convolving effects that makes data interpretation even more challenging as the example shown here. By comparing the relative levels of modifications in subsequent cleavage sites (bar graph), a single solution of positional isomers of two acetyl groups targeting four sites can be determined (A) but impossible for dimethylation in the same scenario (B). Noting that it is not always possible to achieve unambiguous assignment of two acetylation in four targeted sites. If %2ac in cleavage site 3 > %2ac in cleavage site 2, MS^2 is not enough to reach a single solution.

1. Pesavento, J.J., C.A. Mizzen, and N.L. Kelleher, *Quantitative analysis of modified proteins and their positional isomers by tandem mass spectrometry: human histone H4.* Anal Chem, 2006. **78**(13): p. 4271-80.
2. Phanstiel, D., et al., *Mass spectrometry identifies and quantifies 74 unique histone H4 isoforms in differentiating human embryonic stem cells.* Proc Natl Acad Sci U S A, 2008. **105**(11): p. 4093-8.
3. Feller, C., et al., *Global and specific responses of the histone acetylome to systematic perturbation.* Mol Cell, 2015. **57**(3): p. 559-71.