Inference of H3S28ph

Our GCP (Global Chromatin Profiling) analysis did not include the H3S28ph variants on the H3 peptide spanning residues 27-40. Thus, we were unable to claim with our typical high confidence level that H3S28ph is present in the samples. Nonetheless, the GCP assay did provide us with the following evidence to allow us to infer the presence of this modification:

We noted a general trend of decrease of nearly all forms of the peptide spanning H3 27-40 in the synchronized (mitosis) populations relative to the unsynchronized (interphase) population (**Fig. 2A**). As this observation contradicts the laws of mass conservation, we hypothesized two possibilities: (***i***) H3.1 was being replaced by H3.3, which has a single amino acid substitution (A->S) at residue 31; or (***ii***) a PTM not explicitly monitored in the assay was being deposited on this peptide.

We investigated both possibilities. Our assay included a proxy peptide for histone H3.3 – H3.3K27me0K36me0. This proxy peptide presented the same trend as the H3.1K27me0K36me0 peptide, and thus ruled out the option that H3.1 was replaced by H3.3.

We next evaluated additional post-translational modifications. H3S28ph is a known mark that occurs at low frequency but is associated with mitosis. We searched for precursor *m/z* values that would be consistent with the presence of H3S28ph. Indeed, we were able to detect these species based on co-eluting isotopes with the same *m/z* as the expected H3S28ph forms of H3K27me0K36me0, H3K27me1K36me0, H3K27me2K36me0, H3K27me3K36me0, and H3K27ac1K36me0. In each case the, the modified peptide was detected with a charge state of 3 and an adduct mass of 79.966, or *m/z* of 26.655 at *z*=3. **Fig. I** provides an example detection of H3K27me0S28ph1K36me0 at *m/z* 579.9731, *z*=3:

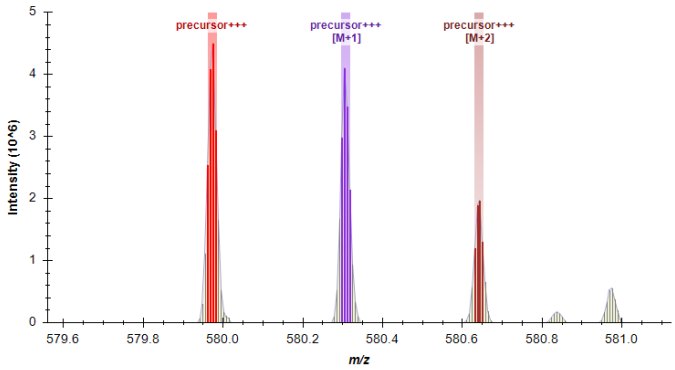

Figure I. Detection of H3K27me0S28ph1K36me0 by co-elution of isotopes. Representative example of detecting a precursor *m/z* consistent with the presence of H3S28ph1. Grey peaks are directly observed from MS data. Red, purple, and brown overlays represent the calculated *m/z* values of the $[M\ 3H]^{+++}$, $[M+1\ 3H]^{+++}$, and $[M+2\ 3H]^{+++}$ ^{13}C isotopes of the H3K27me0S28ph1K36me0 peptide, respectively.

Thus, this provides a strong circumstantial evidence that the observed modification is H3S28ph. Moreover, in line with the increase of H3S28ph in mitosis, we identified higher levels of the inferred H3S28ph in the MS data in the synchronized (mitosis) state relative to the unsynchronized (interphase), basically consistently going up irrespective of other marks present on the peptide.

In some cases, we may not accurately exactly assign the K27 and K36 marks (*i.e.*, the K27me1S28ph1K36me0 peptide could actually be K27me0S28ph1K36me1). Yet, we are quantifying the same analytes in all the samples as judged through matching retention times in LCMS (liquid chromatography mass spectrometry).

Of note, we do not expect the reduced confidence we have in detecting H3S28ph to have impact on our estimates of the total population measurements of H3 (**Fig 2B**) since our calculations are done using the known concentrations of the reference standards added to the samples. Further, only marks containing K27 and/or K36 would be affected by the presence of H3S28ph. Additionally, we cannot incorporate the precursor-based measurements of the H3S28ph species into the stoichiometry calculations. Different peptides have different ionization efficiencies, and thus we cannot compare raw intensities from peptide to peptide directly. We remind the reader that these are estimates of the global stoichiometry. In a similar manner to S10ph, the S28ph modification appears to be present at low abundance in unsynchronized cells, but is wholesale shifted to high abundance during mitosis, irrespective of other modifications present (**Fig II**, compare the shift in pattern from line to line). Thus in analogy to marks quantified on K9 and K14 – where we do have the correct synthetic peptides to fully account for phosphorylation of S10 – we expect the estimates of abundance of marks present on K27 and K36 to be relatively unaffected by the presence of the inferred S28ph-containing species of the H3 27-40 peptide. Note that H3K27ac is parsimonious with K9ac and K18ac, and that the repressive mark K27me3 is unchanged, similar to the repressive mark K9me3.

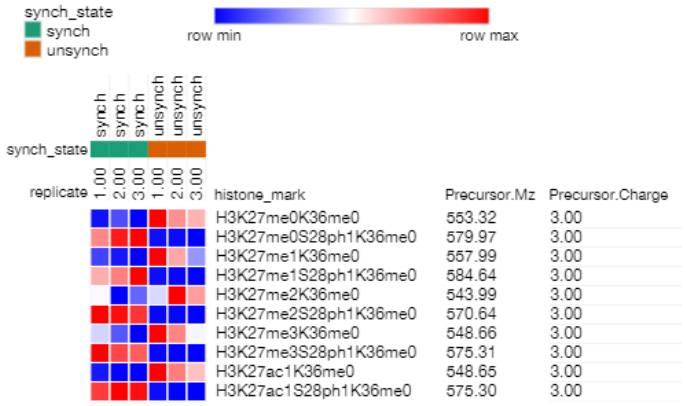

Figure II. Estimates of relative H3S28ph1 levels in synchronized (mitosis) and unsynchronized (interphase) samples. Each column is an individual sample, each row is a combination of histone marks on the H3 peptide spanning residues 27-40. The cells are colored relative to each row's minimum and maximum. For purposes of this analysis, all quantifications were performed using integrated peak area for observations of the precursor *m/z* of the peptides indicated in the "histone_mark" annotation. The stark shift in pattern from row to row indicates a strong and consistent alternation of the H3S28ph1 state between the synchronized and unsynchronized states, uniformly across all peptides containing H3S28ph1.