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Supplemental information

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Christopher D. Reinkemeier and Edward A. Lemke

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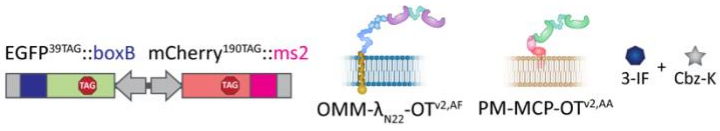
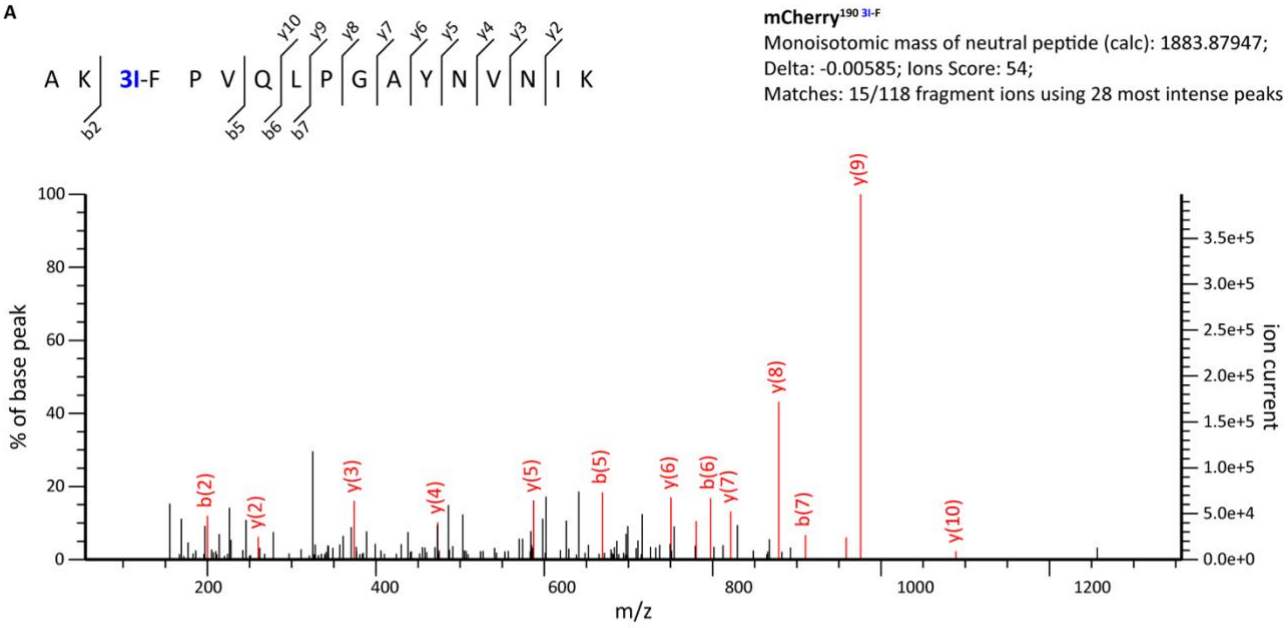
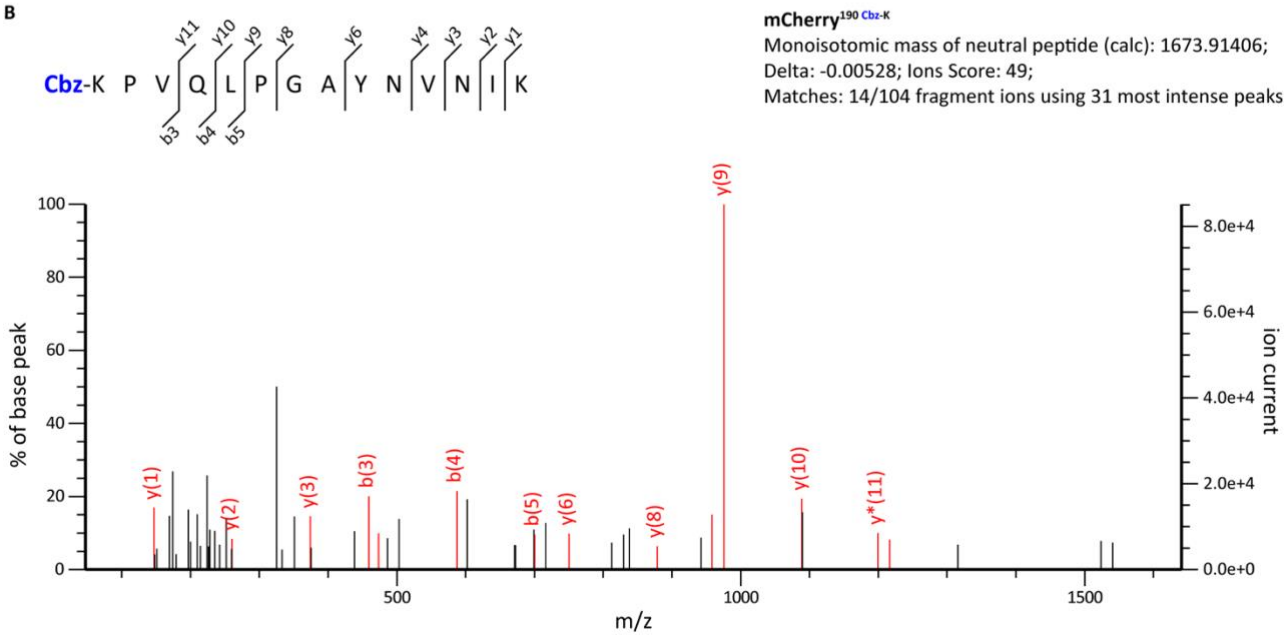
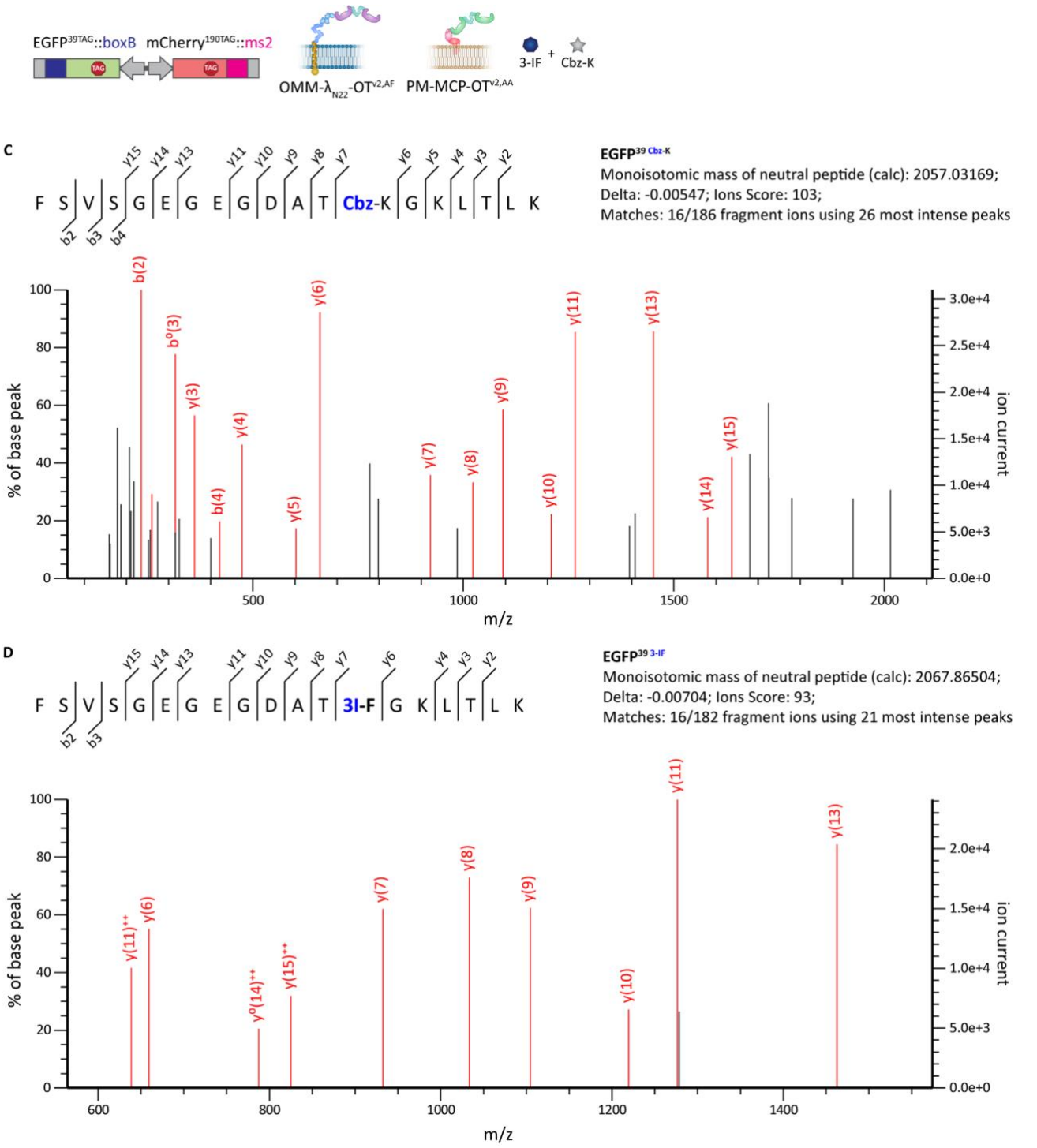
Data S1: Related to Results and STAR METHODS. MS/MS Spectra of mCherry and EGFP purified from Mammalian Cells with Cbz-K and 3-IF.









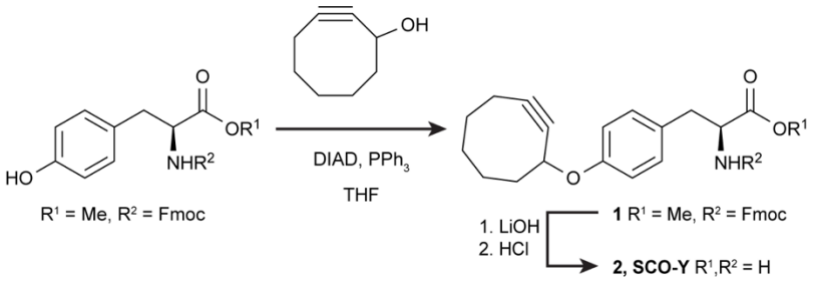
MS/MS spectra of mCherry and EGFP purified from HEK293T cells expressing OMM-λ_{N22}-OT^{v2,AF} system together with PM-MCP-OT^{v2,AA}, the double-recruitment reporter (EGFP^{39TAG}::boxB, mCherry^{190TAG}::ms2) and tRNA^{Pyl} in presence of the ncAAs Cbz-K and 3-IF. EGFP and mCherry were co-purified using a mix of equal amounts of GFP- and RFP-Trap beads and subsequently analyzed via SDS-PAGE, followed by tryptic digest in the gel and LC-MS/MS analysis.

(A,B) By design mCherry should be modified at position 190 (see Table S4 for sequence details) and it should be predominantly modified with 3-IF, thus we can identify peptides corresponding to mCherry$^{190\ \mathbf{3\text{-}IF}}$ (A). However, from FFC analysis we know that for this system we expect a 6-fold selectivity for incorporating a phenylalanine derivative into mCherry over EGFP and a 7-fold ratio for incorporating a lysine derivative into EGFP over mCherry (see Figure 5). Thus, also while most mCherry molecules should contain 3-IF some can also contain Cbz-K. As LC-MS/MS is highly sensitive, we can thus also identify peptides corresponding to mCherry$^{190\ \mathbf{Cbz\text{-}K}}$ (B).

(C,D) Analogously, EGFP should be modified at position 39 (see Table S4 for sequence details) and it should be predominantly modified with Cbz-K and we can identify peptides corresponding to EGFP$^{39\ \mathbf{Cbz\text{-}K}}$ (C). However, also here we expect the effect of finite selectivity (as described above) and thus we can also detect EGFP$^{39\ \mathbf{3\text{-}IF}}$ (D), which is in line with our expectation that while most EGFP molecules should contain Cbz-K some can also contain 3-IF, which can be detected by a LC-MS/MS due to the very high sensitivity.

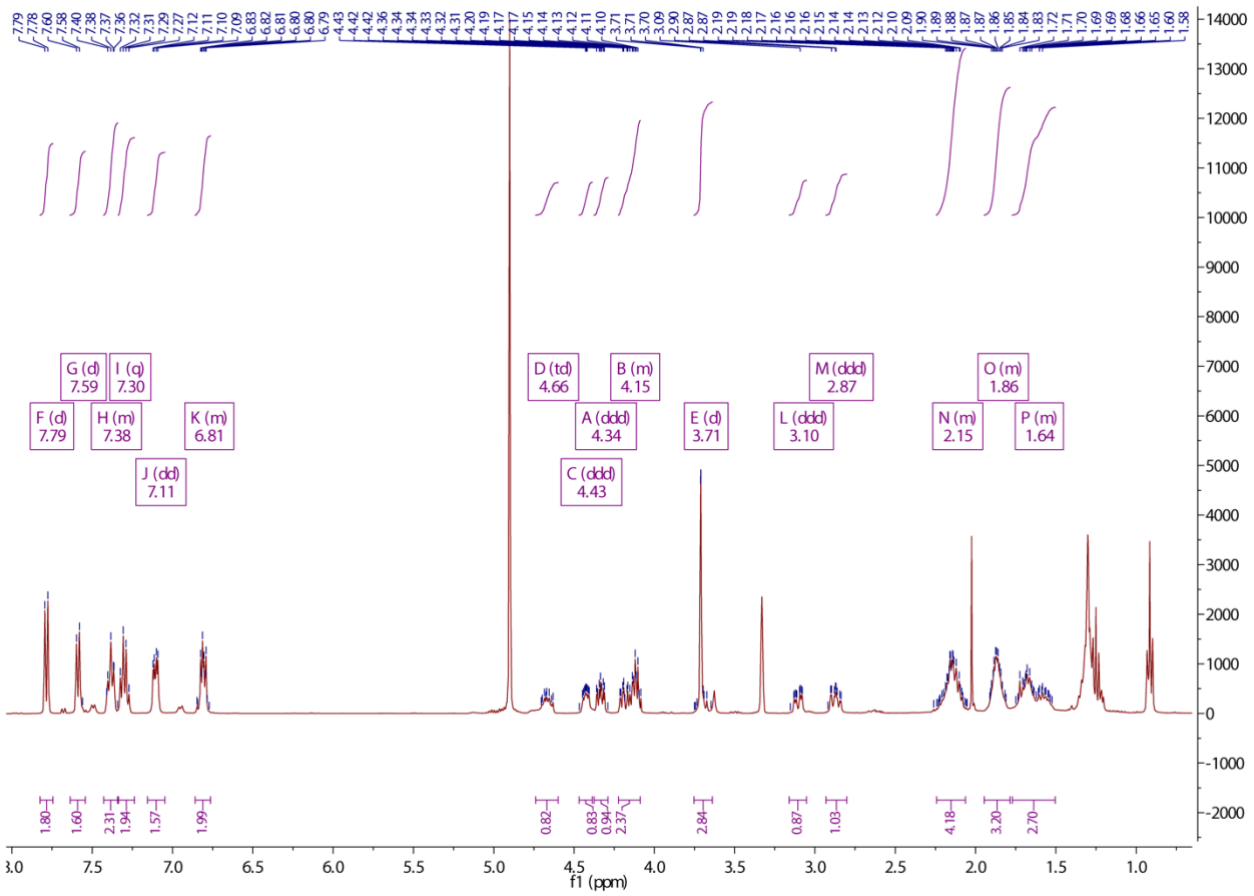
It is however important to note that from these non-quantitative MS/MS experiments we cannot deduce a ratio of how much 3-IF or Cbz-K is incorporated in each respective protein and thus we performed bioorthogonal labeling experiments to get a more quantitative estimate as shown in Figure 6. Here, we show spectra for peptides with the highest detected “Ions Score”.

Data S2: Related to STAR METHODS. Synthesis Scheme for SCO-Y and corresponding NMR spectra.

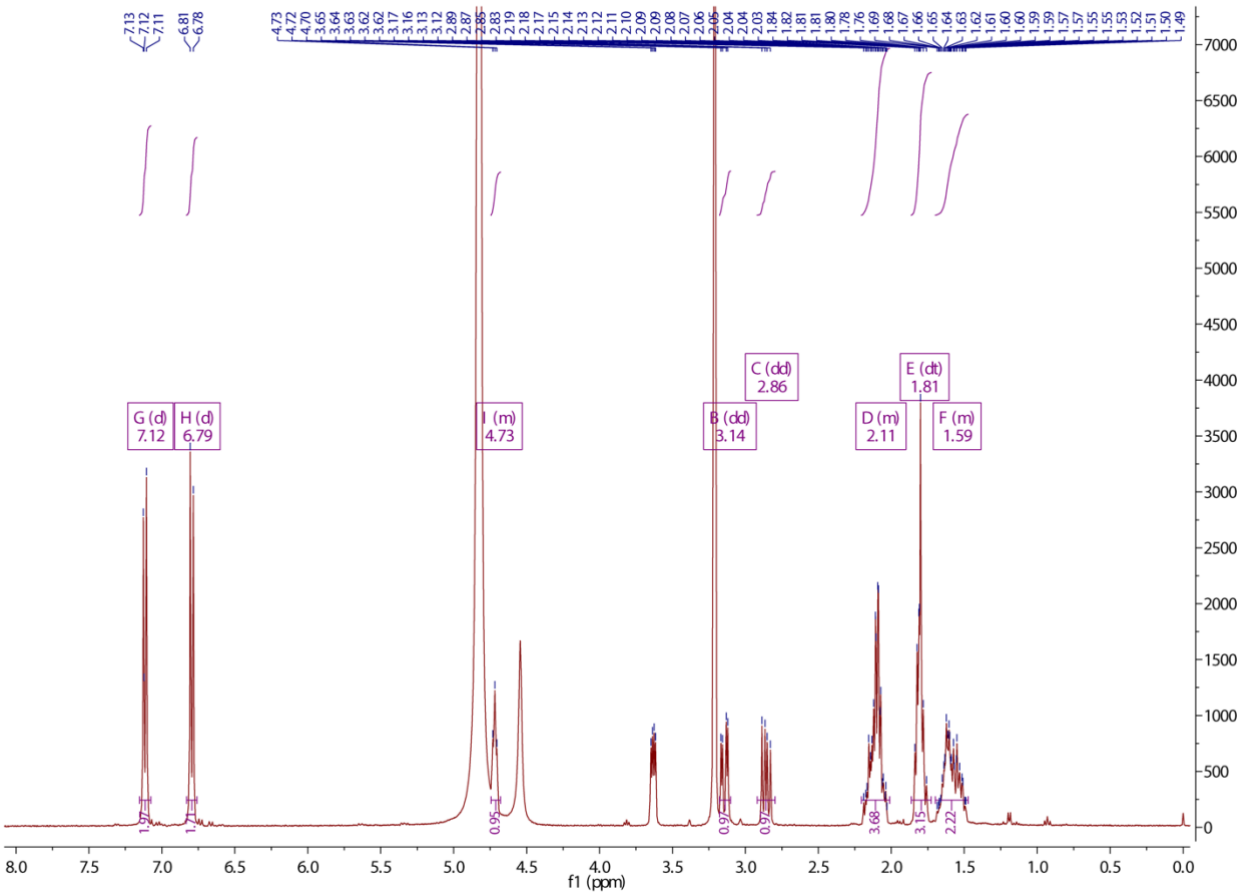


Synthesis scheme SCO-Y as described in the STAR METHODS section.

^{1}H NMR of Fmoc-SCO-Y-OMe (**1**)



^{1}H NMR of SCO-Y (**2**)



^{13}C NMR of SCO-Y (**2**)