Supplementary Information (SI)

List of SI materials

Table S1. The relative amount of EGFR phosphor-peptides in HEK293 T-REx (HEK) cells and HEK-EGFR T-REx cells with indicated treatment.

Table S2. EGF induced EGFR associated proteins.

Table S3. The EGF induced different EGFR associated proteins between wild type EGFR (WT) and Y998A variant.

Table S4. The anisomycin induced different EGFR associated proteins between wild type EGFR (WT) and 4A mutant.

Legends for SI Figures

SI Figure S1. Quantification of EGFR in HEK-EGFR T-REx cells by WB and SRM. (A) Quantification of WB results from Figure 1. Error bars denote standard deviation from 3 independent biological replicates. (B) SRM ion currents of EGFR peptide (IPLENLQIIR) with a spiked-in, heavy stable isotope-containing standard peptide of identical sequence.

SI Figure S2. Immunostaining of EGFR for HEK-EGFR T-REx cells. (A) Bright field (BF) images of the indicated HEK-EGFR T-REx cells, which correspond to Figure 2. (B) Immunostaining of EGFR in HEK-EGFR T-REx cells following incubation at 4 °C for 1 h with vehicle (Cont4C) or 10 μM anisomycin (Ani4C). (C) Immunostaining of EGFR (red) and Rab4A (green) as a marker of recycling and degradative endosomal compartments. Cells were treated without (Cont) or with EGF at 37 °C for 15 min (EGF37C). EGF-stimulated colocalization of EGFR and Rab4A in an endosomal compartment is indicated by the punctate yellow features in the EGF37C but not Cont sample. Nuclei were stained blue by using DAPI. Scale bars are 7 μm.

SI Figure S3. Comparison of EGFR associated proteins before and after internalization as a function of cell treatment with EGF ligand and stress (anisomycin). (A) Volcano plot comparing EGFR associated proteins identified by AP-MS in cells expressing WT EGFR after treatment EGF at 37 °C and detected in with control HEK293 cells lacking EGFR-flag expression. The $\log_2$ ratio of protein intensities was plotted against negative $\log_{10}$ *p*-values (n=3). Proteins with $p < 0.05$ are positioned above the horizontal dashed line, and differential proteins defined as $|\text{Log}_2\text{Ratio}| > 6$ are fall outside the narrow range defined by the two vertical dashed lines. Selected differential proteins are indicated by red dots and annotation with the protein name. (B) Heat map depicting clustering of 74 EGFR-associated proteins as a function of the indicated cell treatments. MaxQuant was used to quantify these proteins and Perseus software was used to process and cluster the data. The position of EGFR is indicated by the arrow. Black boxes are drawn around major clusters associated with the indicated treatments, and defined by the adjacent dendrogram.

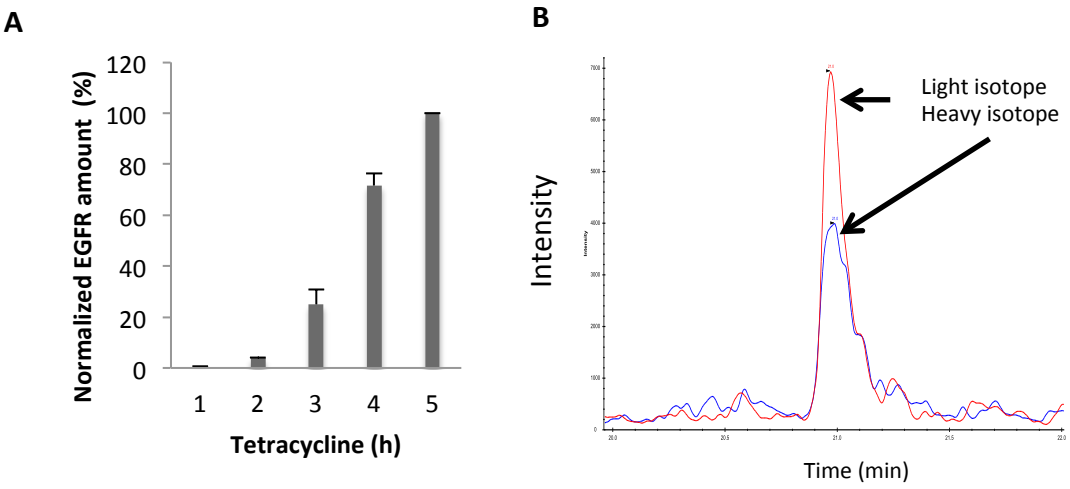
SI Figure S4. Comparison of expression, phosphorylation, and interactome of WT and variant EGFR. (A) WB analysis of whole cell lysates from HEK-EGFR T-REx cells with indicated antibody. Tet inducible HEK293 T-REx cells were stably transfected with wild type or variant-encoding EGFR-Flag pcDNA vectors and then treated without (No Tet) or with Tet (Tet; 1 μg/ml, 24 h). Two clonal stable cell lines, labeled 1 and 2, were tested for each construct. (B) Clustering analysis EGFR phosphorylation sites associated with the indicated cell treatments and quantified in WT and the indicated EGFR variants. MaxQuant and Perseus were used for phosphopeptide quantification and clustering analysis. (C) Volcano plot showing the different binding partners between WT EGFR and the Y998A variant after EGF treatment at 37 °C (left panel), or between WT EGFR and the 4A variant after anisomycin treatment at 37 °C (right panel). The plot parameters are the same as shown in SI Fig. S3A.

SI Figure S5. Imaging of WT and Y998F variant of EGFR. (A) Bright field (BF) images the indicated HEK-EGFR T-REx cells, corresponding to Figure 5A. (B) Immunofluorescence imaging of

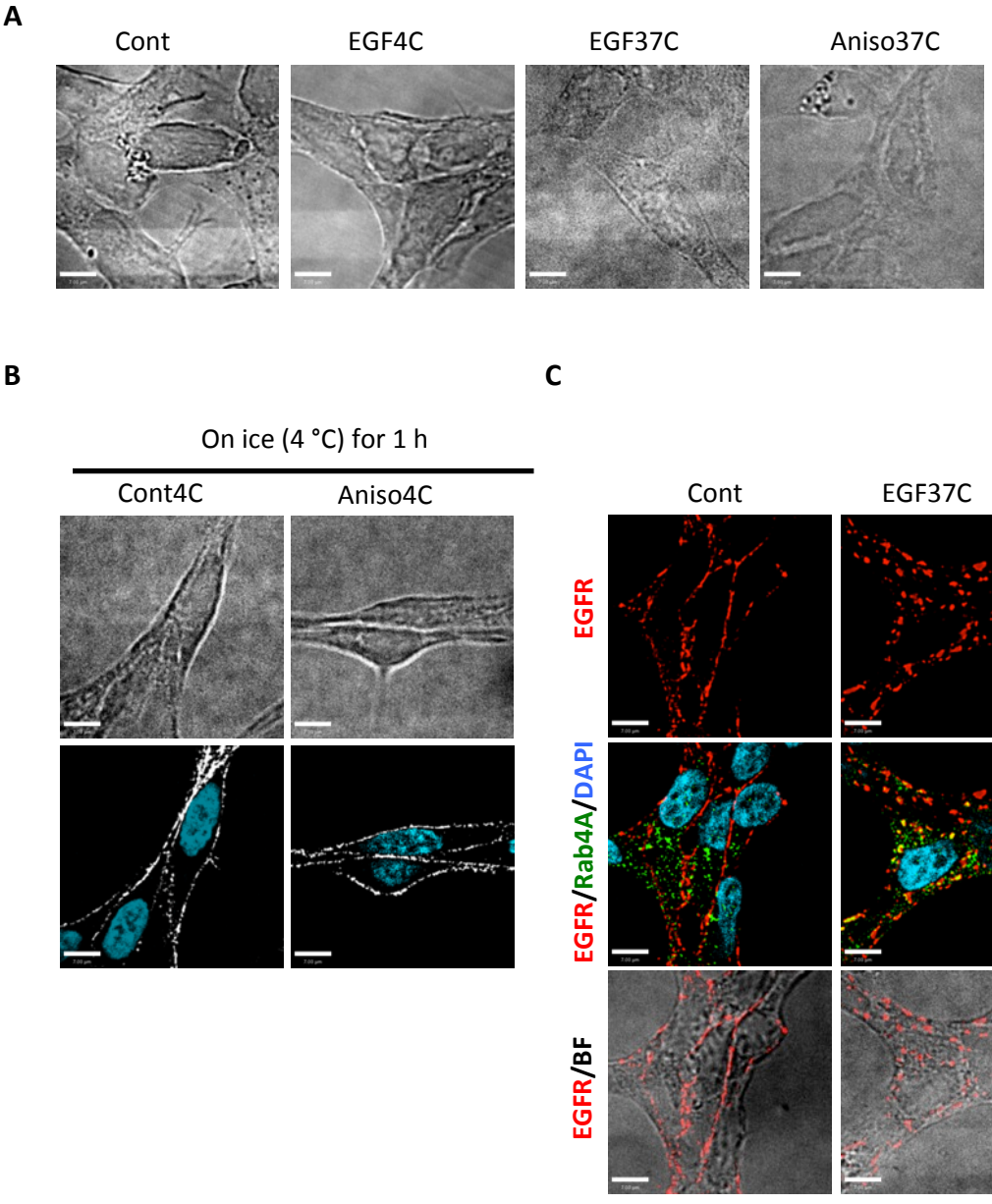
EGFR in HEK cells following EGF treatment with or without 10 μm monensin to block EGFR recycling. (C) Using monensin and biotin labeled EGFR to indirectly measure EGFR recycling. EGFR at the cell surface were covalently modified with cleavable biotin. Biotinylated EGFR was captured by streptavidin (SA) beads, and western blotted for EGFR (anti-Flag). When cell surface biotin was removed by application of the cleavage reaction prior to the SA adsorption step (+ cleavage, lane 2), only internalized (i.e. protected from cleavage) biotinylated EGFR was recovered, whereas in the absence of cleavage (- cleavage, lane 1), both internalized and plasma membrane-localized (total EGFR) were captured. Total internalized EGFR was recovered from cells treated with EGF and 10 μM monensin (EGF + Mon + cleavage, lane 3). Internalized EGFR that failed to recycle was determined from the cells treated with EGF without monensin (EGF + cleavage, lane 4). The amount of recycled EGFR was calculated by subtracting signals of lane 4 (internalized EGFR; failed to recycle) from lane 3 (total internalized EGFR).

SI Figure S6. Tandem mass spectra of modified peptides. A series of representative MS/MS spectra are shown, and provide evidence for the identification and localization of modification for each of the measured and quantified phosphorylated and ubiquitinated peptide ions. The y and b fragment ions are indicated, and the sequence of the peptide is shown at the top of each panel. The spectra were produced by using Scaffold software; all MS/MS information is included in SI Table S1.

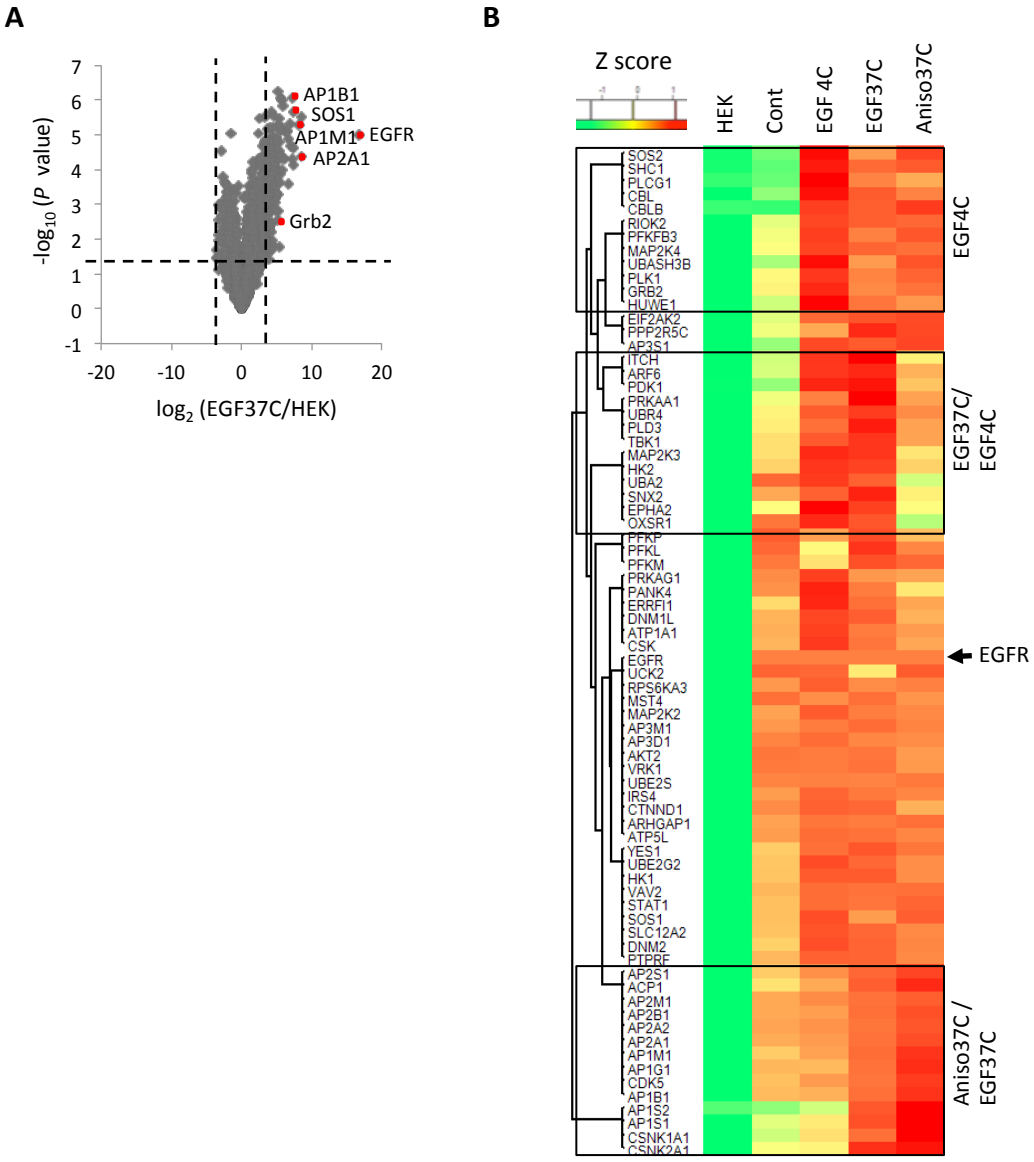
SI Figure S1


A
Normalized EGFR amount (%)
120
100
80
60
40
20
0
1
2
3
4
5
Tetracycline (h)
B
Intensity
Light isotope
Heavy isotope
Time (min)

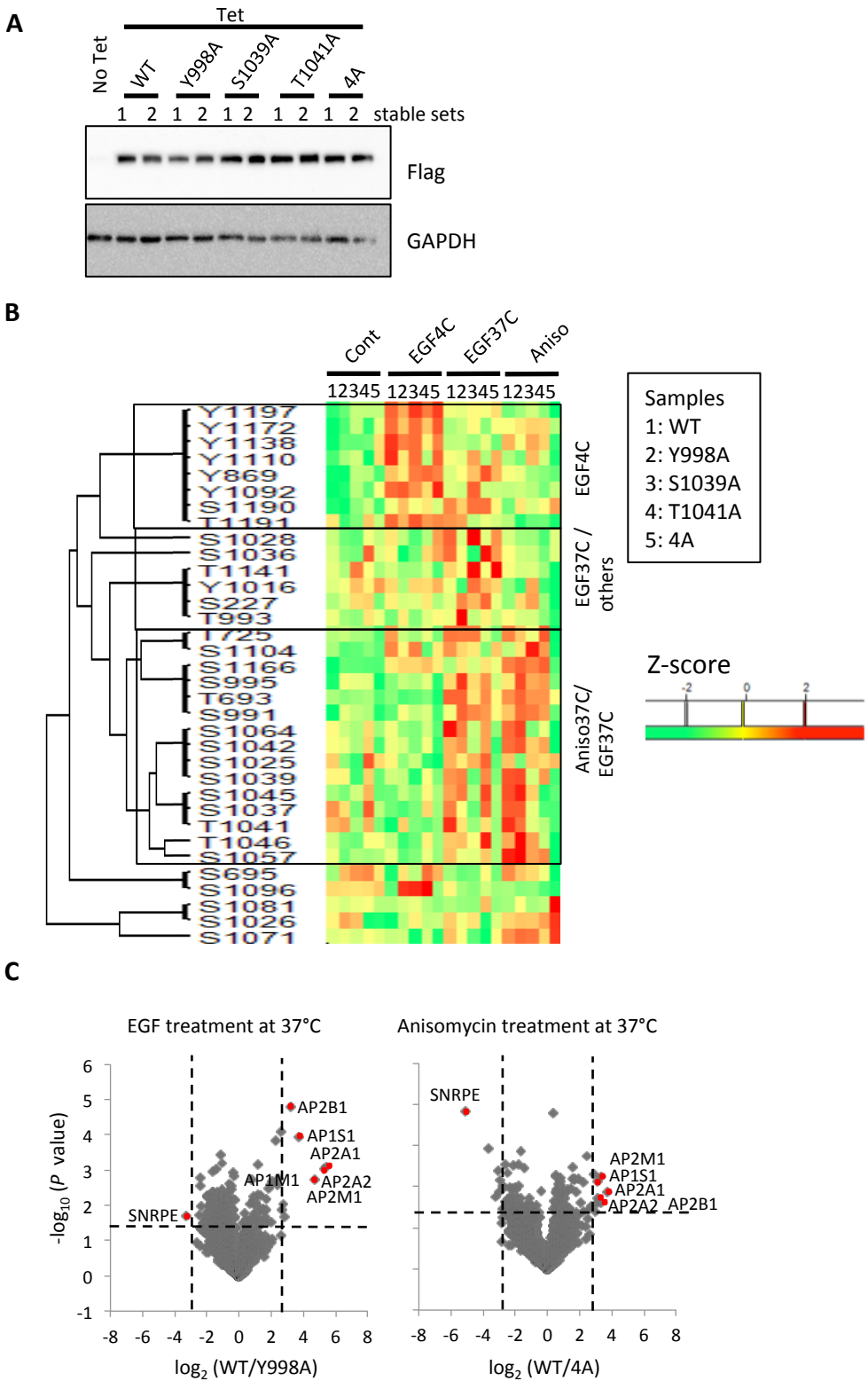
SI Figure S2


A
Cont
EGF4C
EGF37C
Aniso37C
B
On ice (4 °C) for 1 h
Cont4C
Aniso4C
C
Cont
EGF37C
EGFR
EGFR/Rab4A/DAPI
EGFR/BF

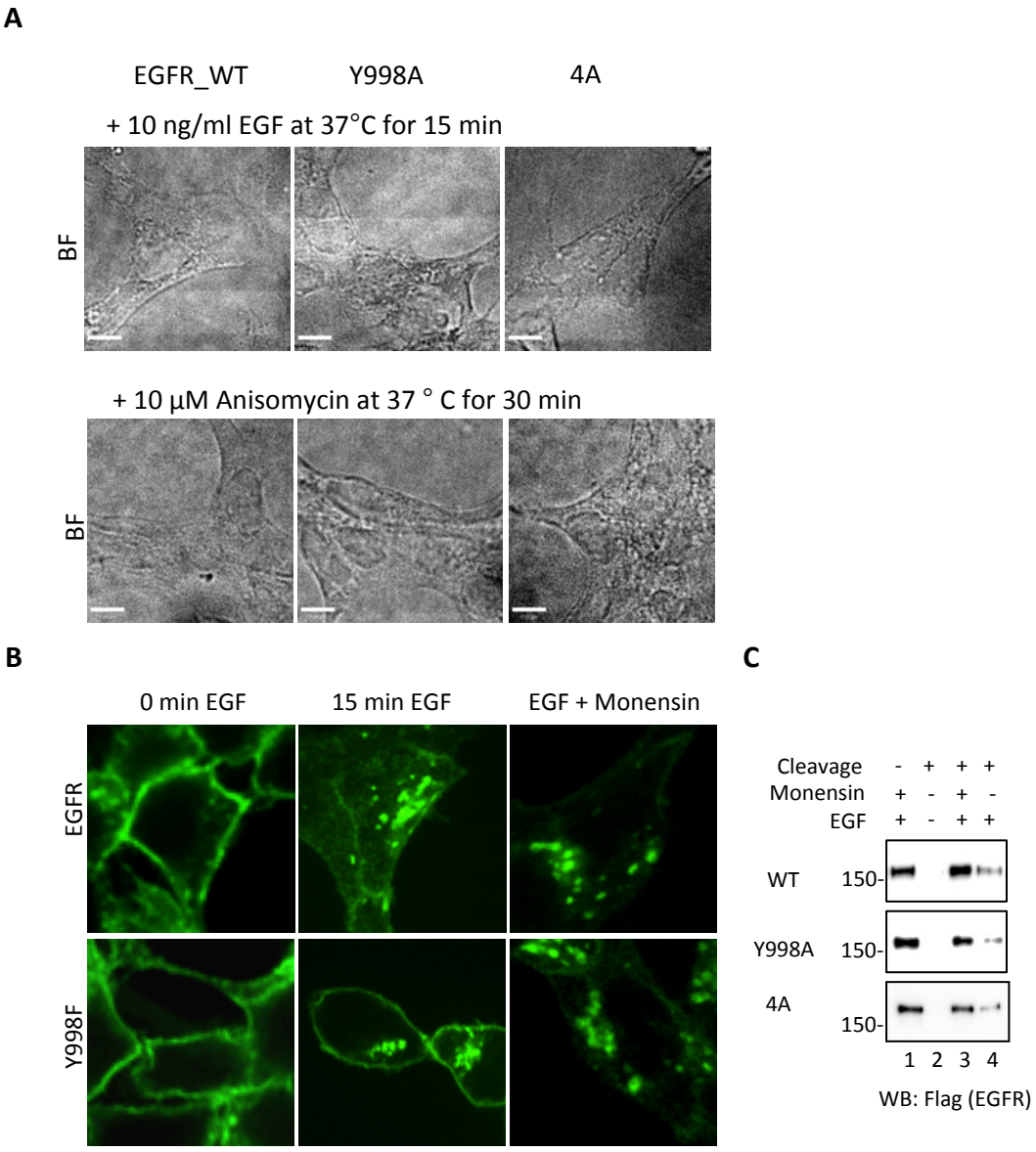
SI Figure S3


A
B
AP1B1
SOS1
AP1M1
EGFR
AP2A1
Grb2
-log10 (P value)
log2 (EGF37C/HEK)
Z score
HEK
Cont
EGF 4C
EGF37C
Aniso37C
EGF4C
EGF37C/
EGF4C
EGFR
Aniso37C /
EGF37C

SI Figure S4

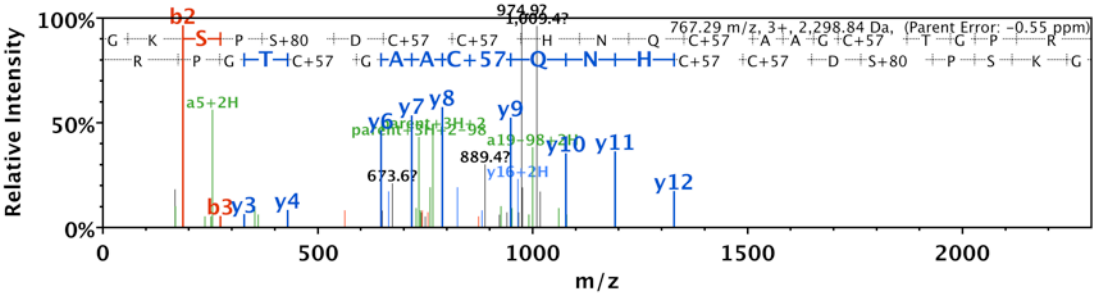

A
Tet
No Tet
WT
Y998A
S1039A
T1041A
4A
1 2 1 2 1 2 1 2 1 2 stable sets
Flag
GAPDH
B
Cont
EGF4C
EGF37C
Aniso
12345 12345 12345 12345
Y1197
Y1172
Y1138
Y1110
Y869
Y1092
S1190
T1191
S1028
S1036
T1141
Y1016
S227
T993
T725
S1104
S1166
S995
T693
S991
S1064
S1042
S1025
S1039
S1045
S1037
T1041
T1046
S1057
S695
S1096
S1081
S1026
S1071
EGF4C
EGF37C / others
Aniso37C/ EGF37C
Samples
1: WT
2: Y998A
3: S1039A
4: T1041A
5: 4A
Z-score
-2
0
2
C
EGF treatment at 37°C
Anisomycin treatment at 37°C
-log10 (P value)
AP2B1
AP1S1
AP2A1
AP1M1
AP2A2
AP2M1
SNRPE
log2 (WT/Y998A)
log2 (WT/4A)

SI Figure S5


A
EGFR_WT
Y998A
4A
+ 10 ng/ml EGF at 37°C for 15 min
BF
+ 10 µM Anisomycin at 37 ° C for 30 min
BF
B
0 min EGF
15 min EGF
EGF + Monensin
EGFR
Y998F
C
Cleavage - + + +
Monensin + - + -
EGF + - + +
WT
150-
Y998A
150-
4A
150-
1 2 3 4
WB: Flag (EGFR)

SI Figure S6

pS229



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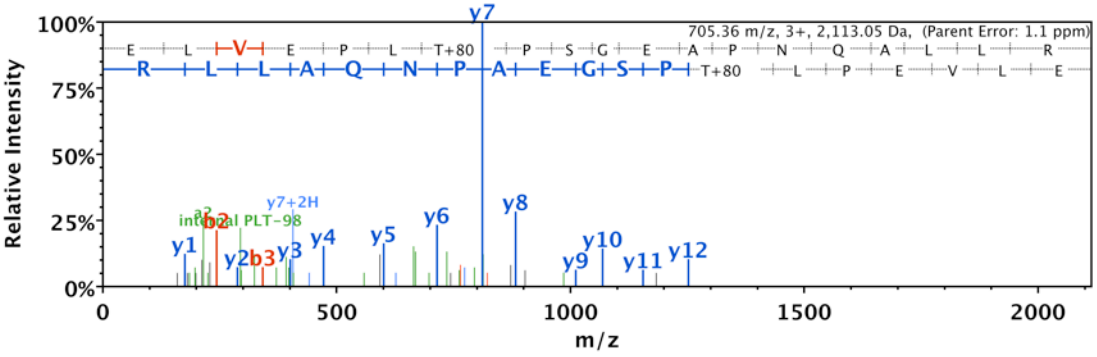

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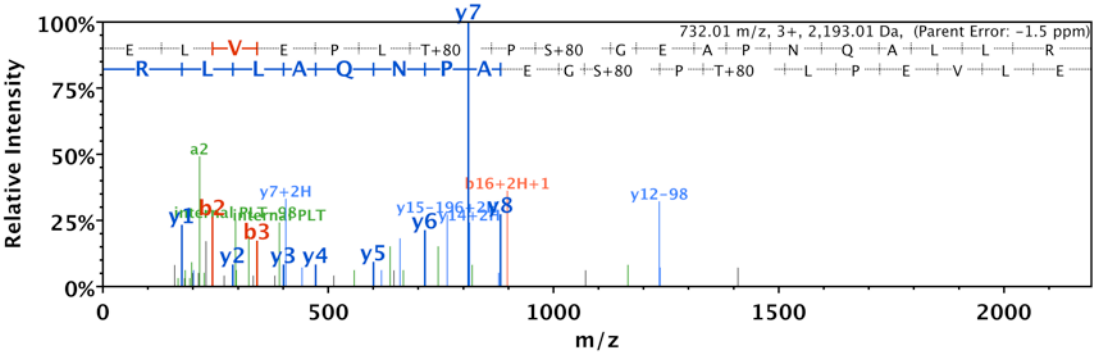

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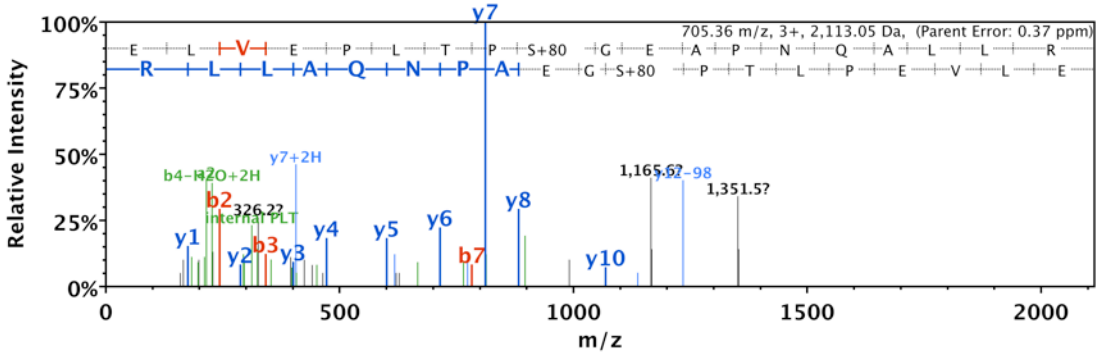

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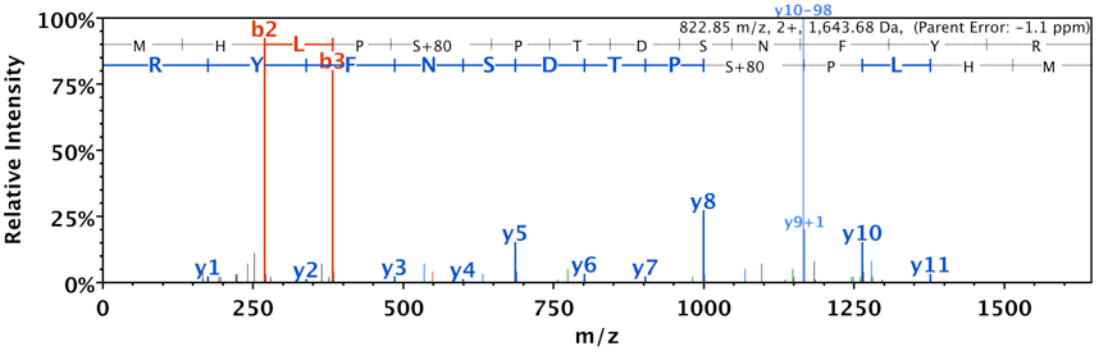

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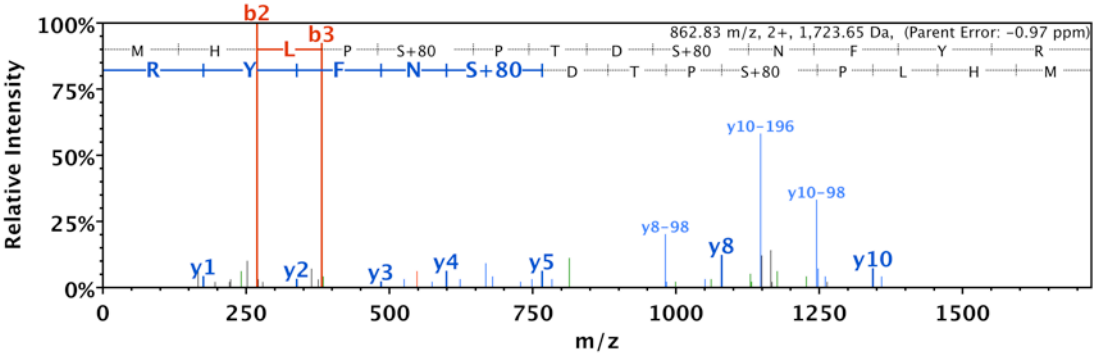

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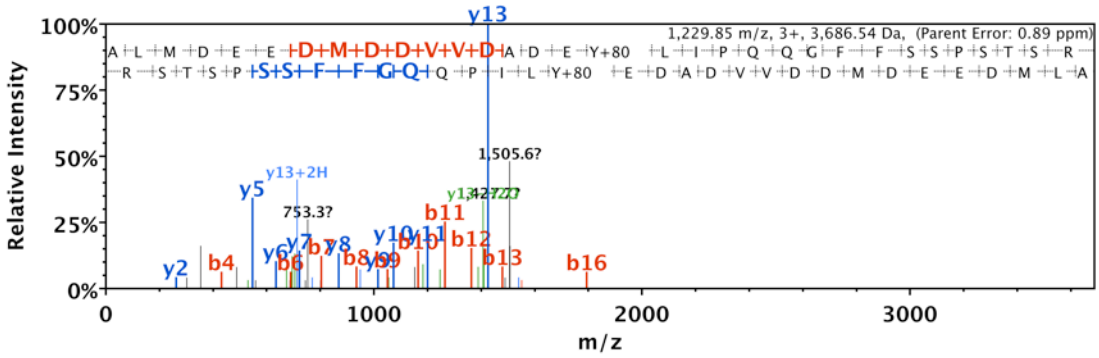

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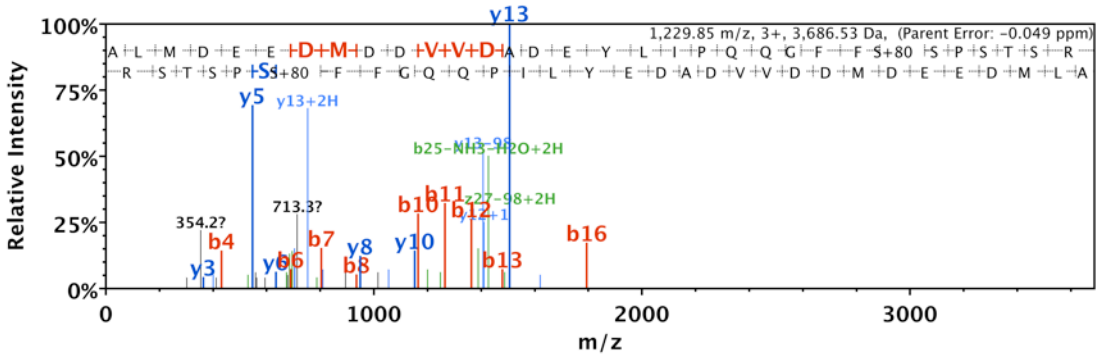

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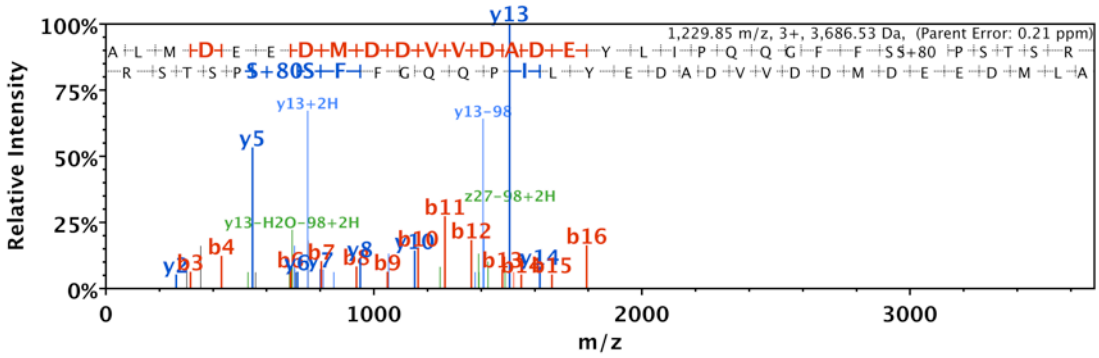

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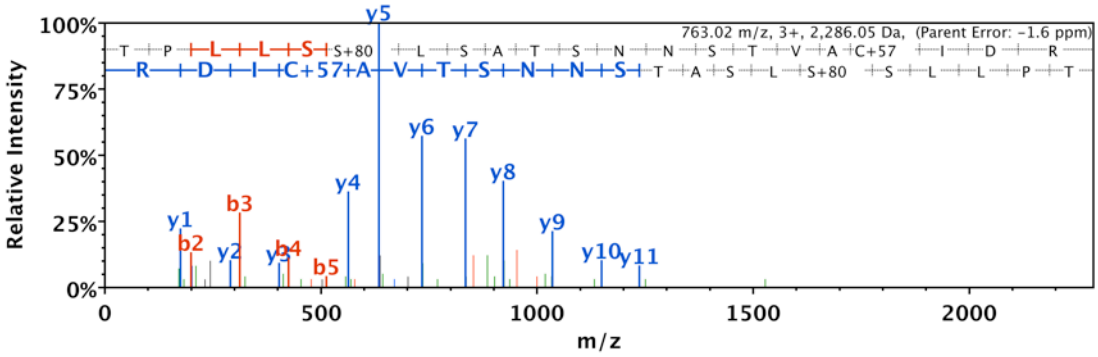

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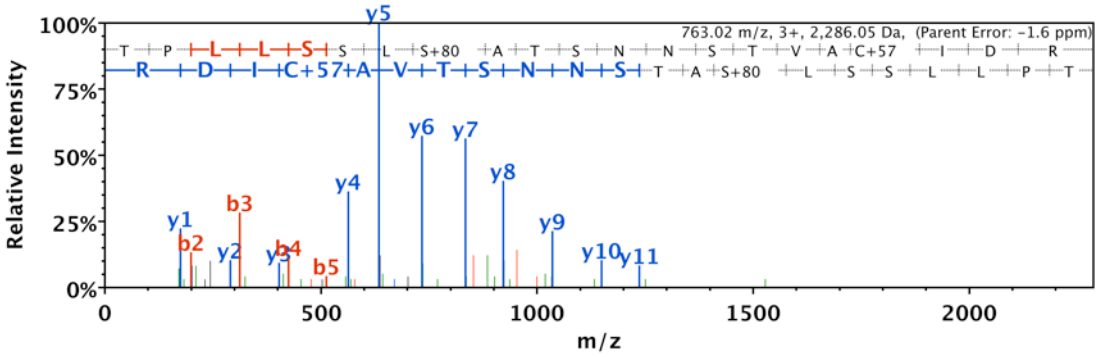

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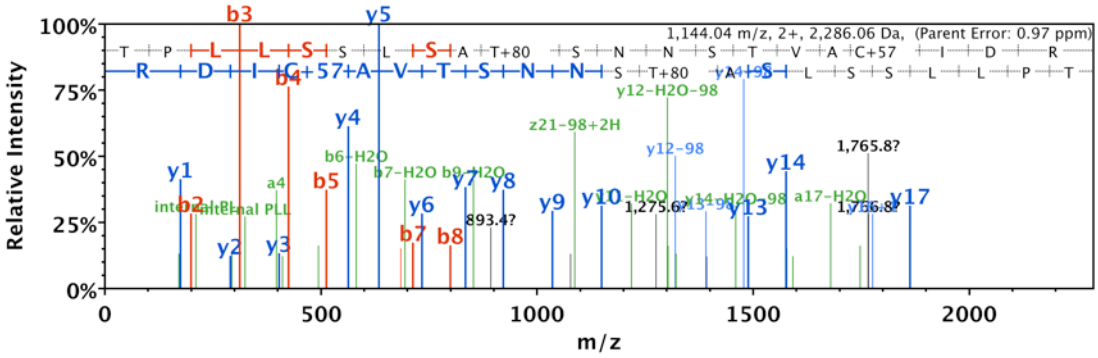

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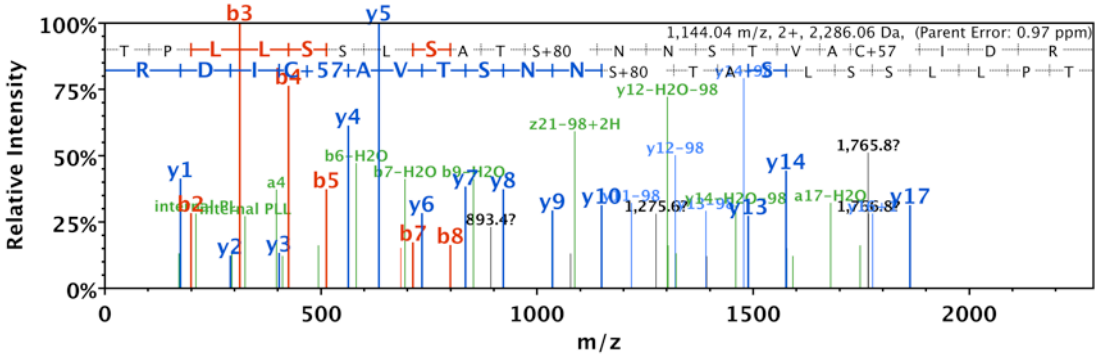
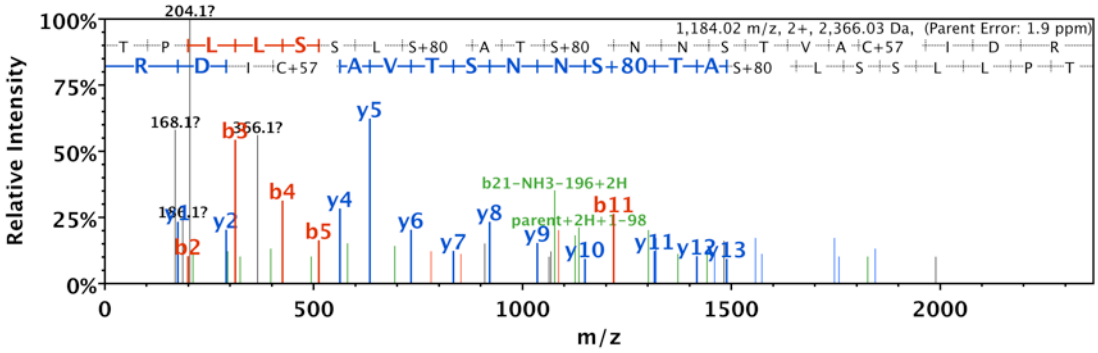
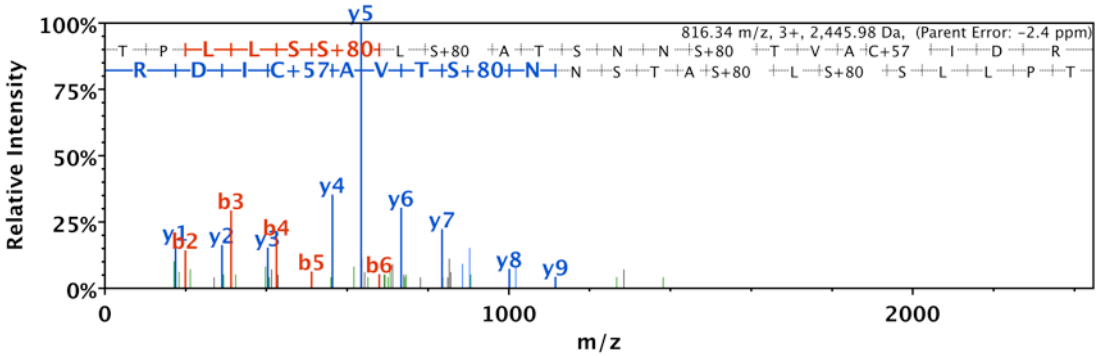


pS1039 & pS1042 or pS1039 & pT1041 or pT1041 & pS1042



pS1037 & pS1039 & pS1045



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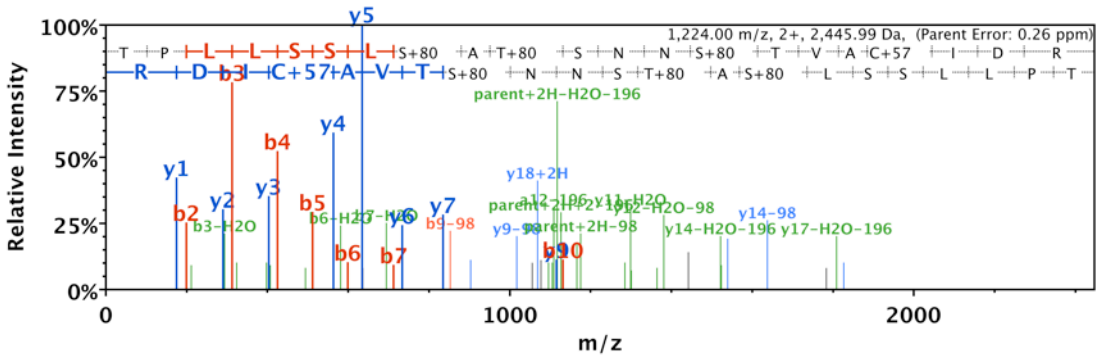

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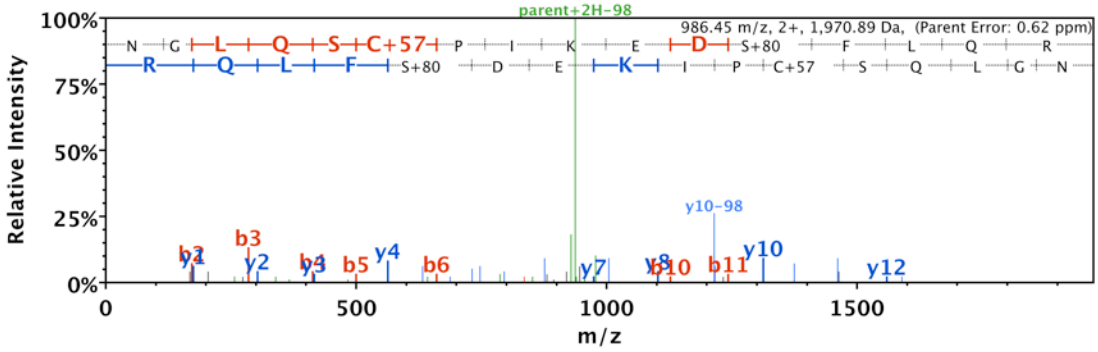

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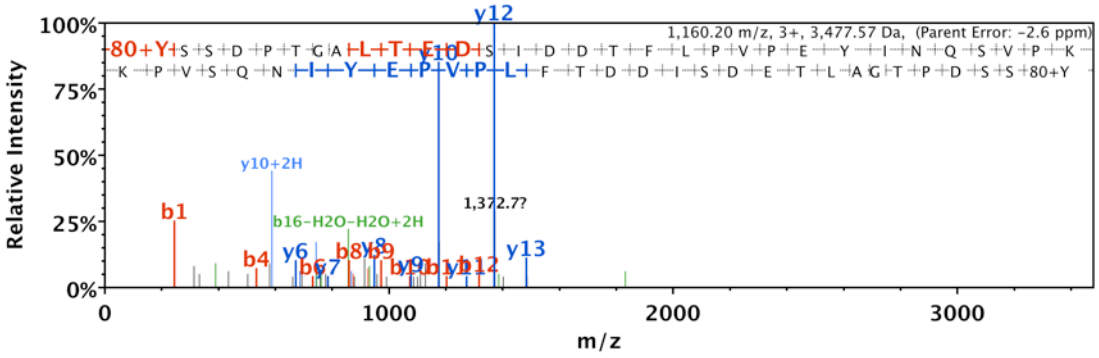

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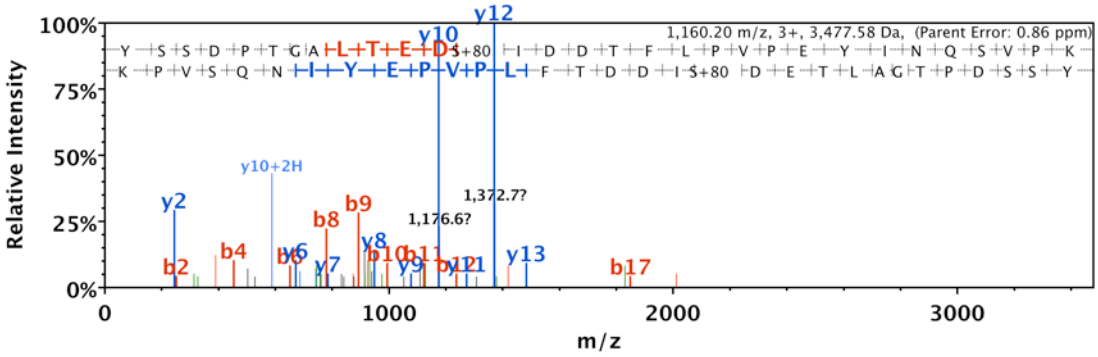

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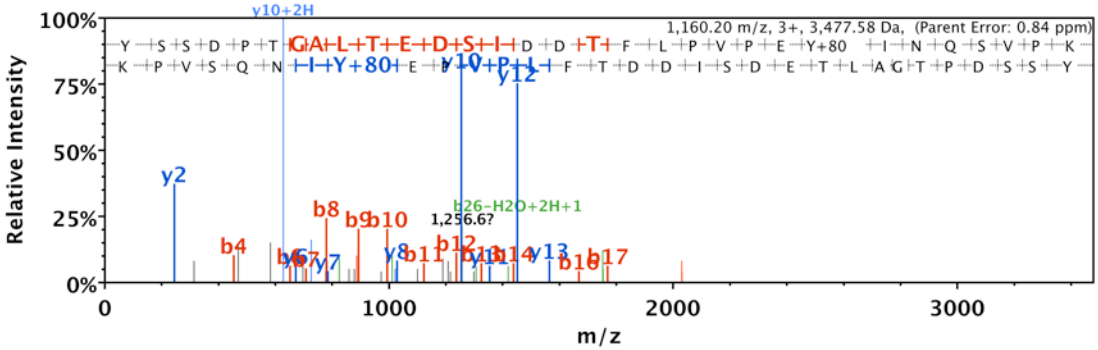

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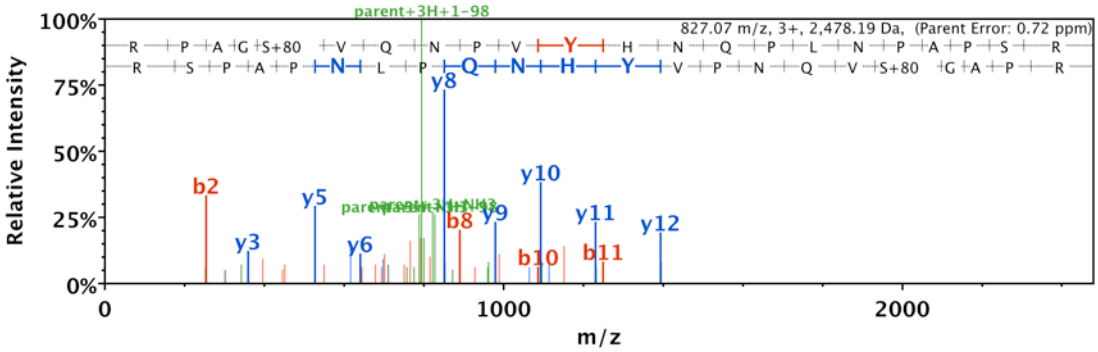

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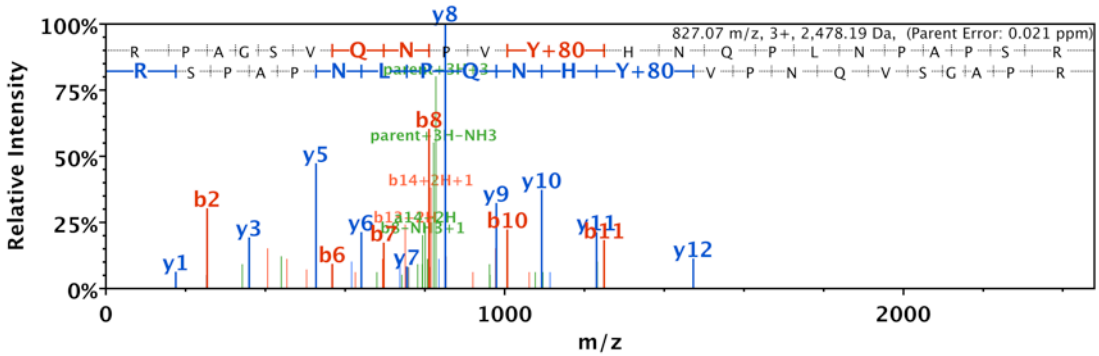

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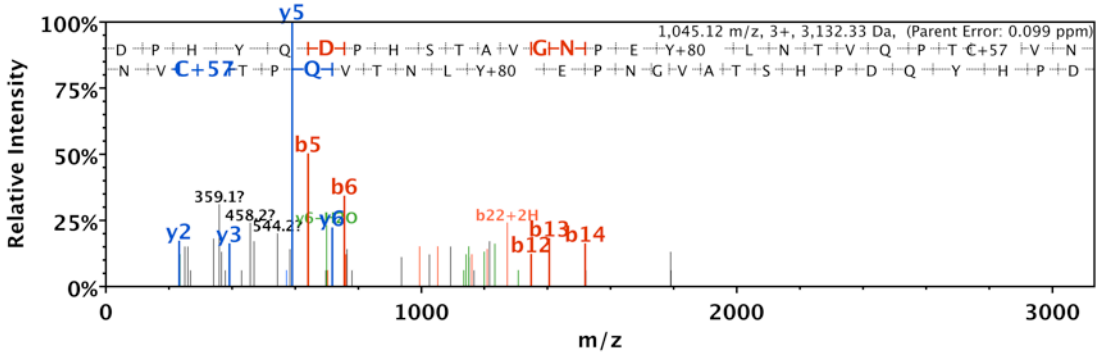

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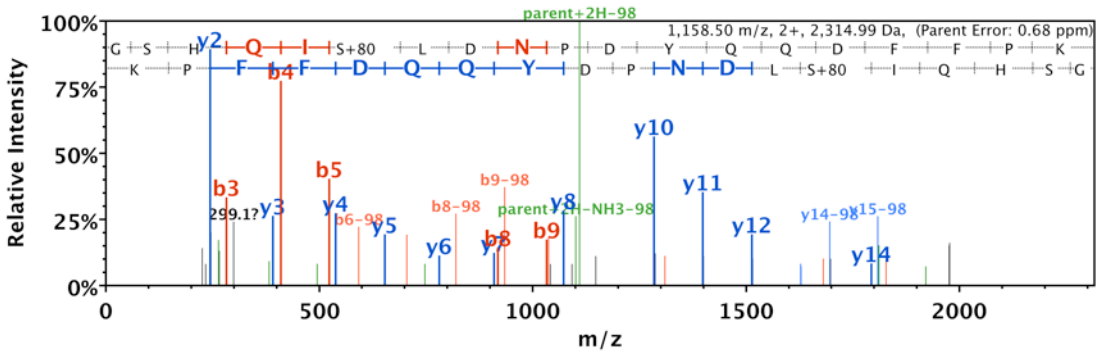

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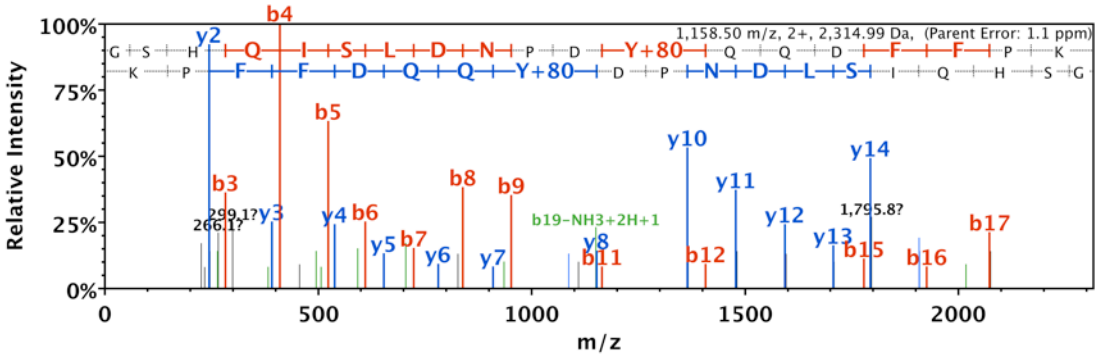

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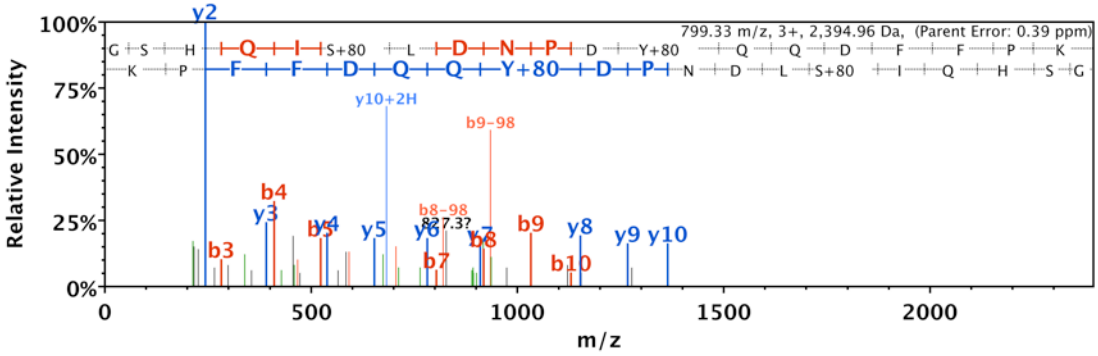

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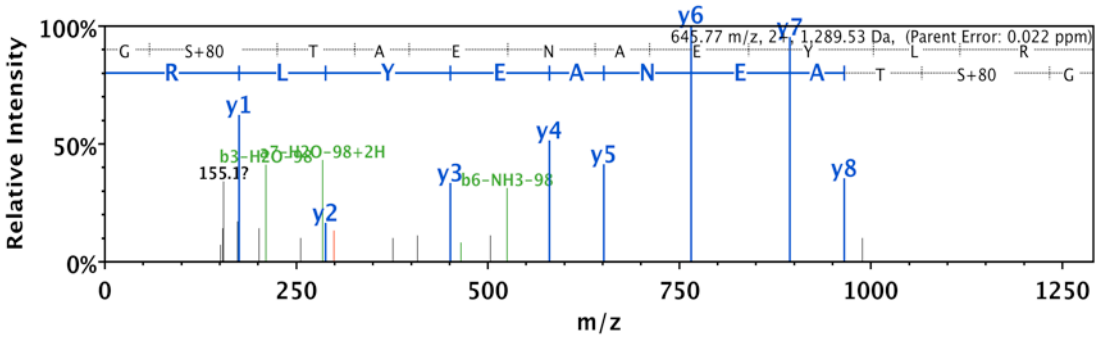

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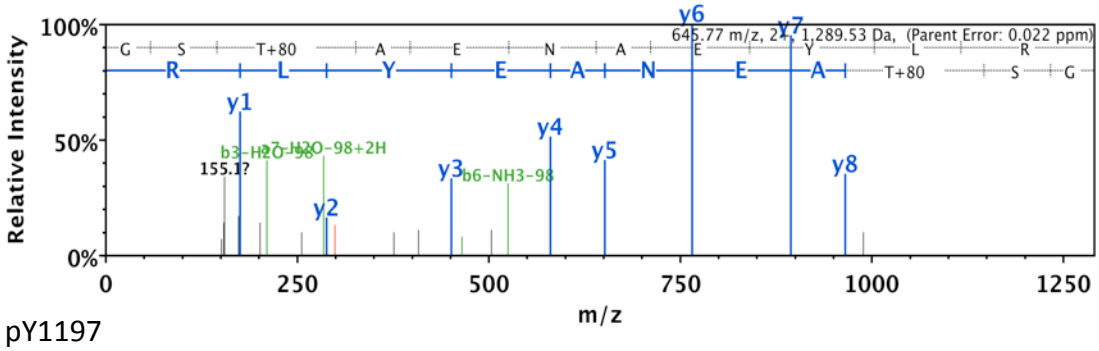

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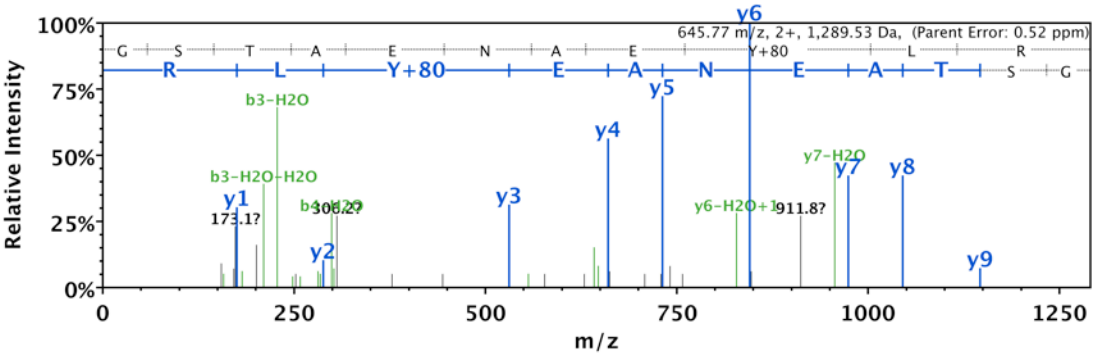
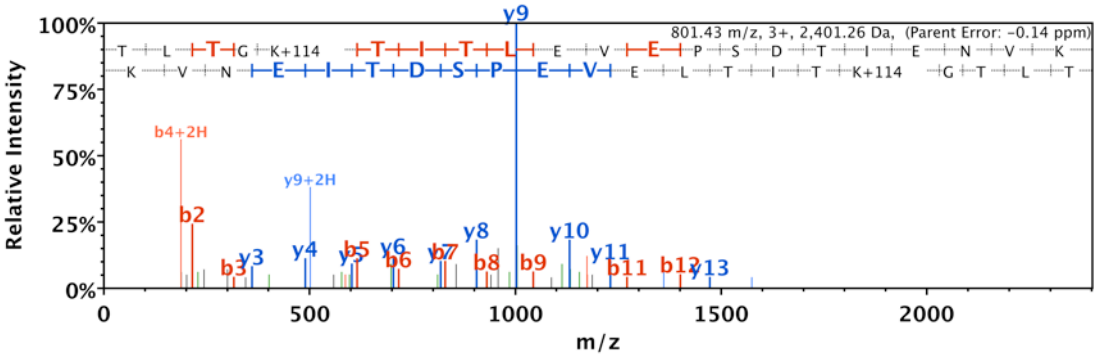
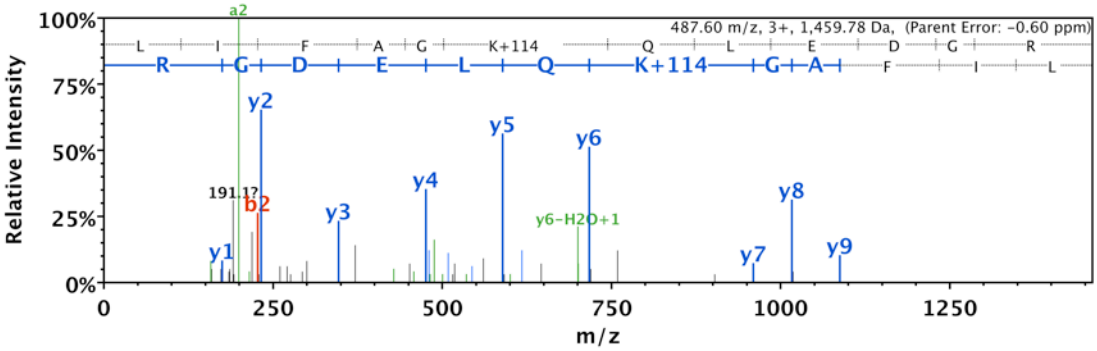


Ubiquitin modification (K-GG)

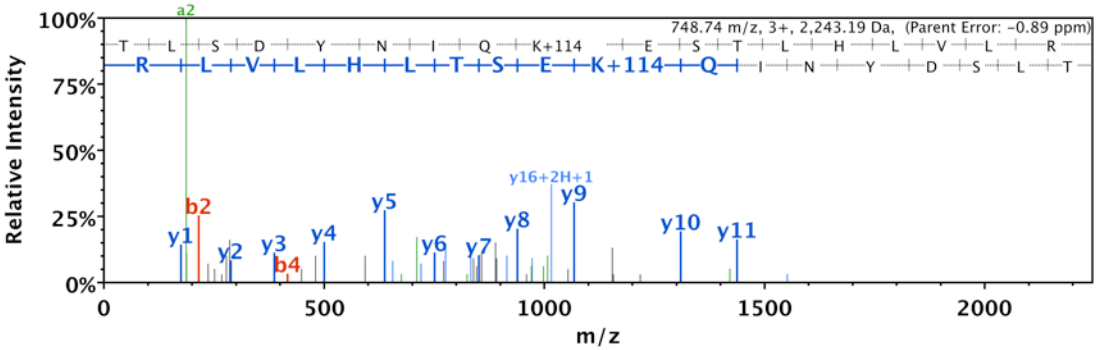
Ubiquitin K11-GG



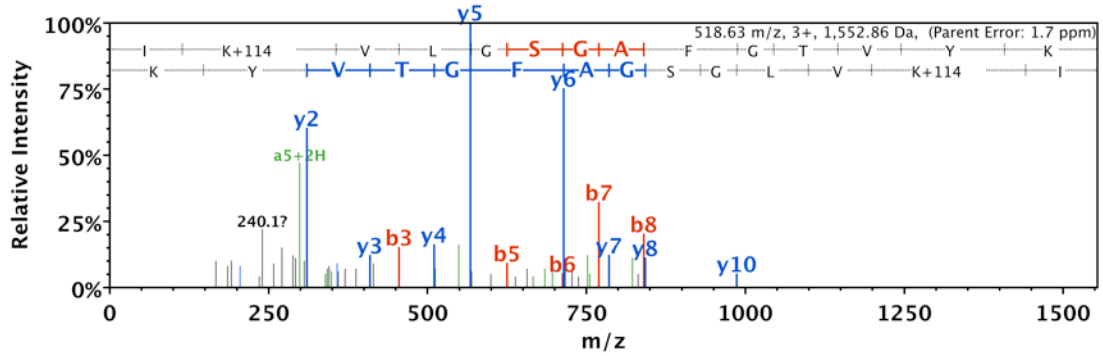
Ubiquitin K48-GG



Ubiquitin K63_GG



EGFR K716-GG



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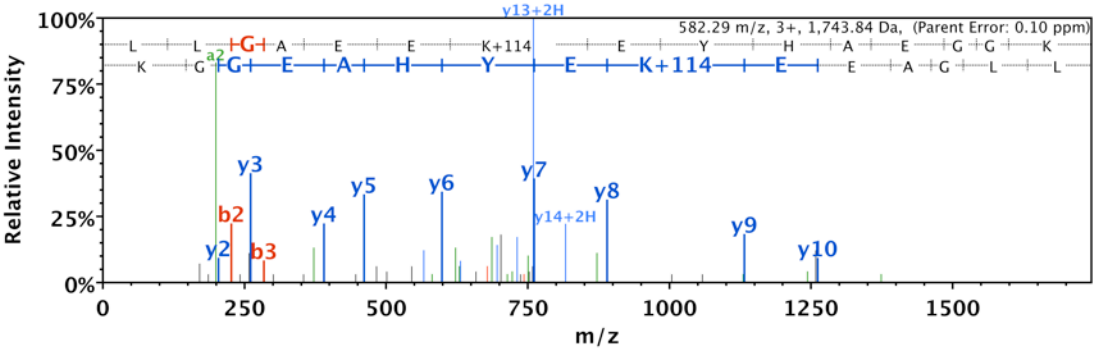

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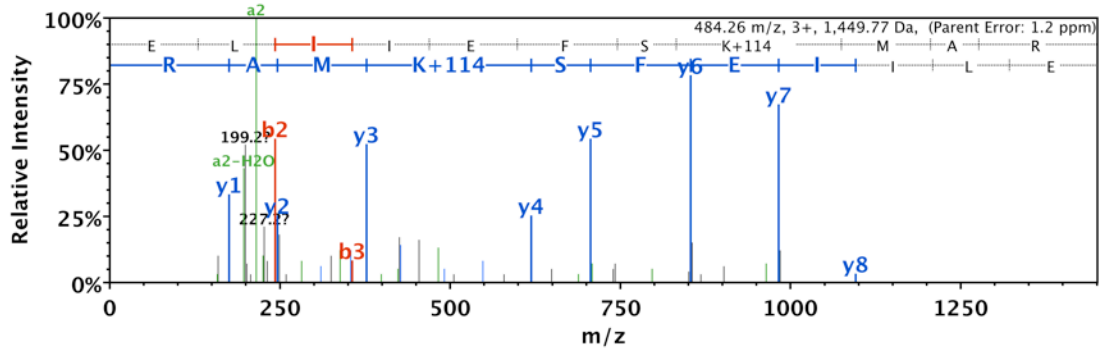

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