Supplemental Experimental Procedures

Plasmid Construction

For the generation of a plasmid encoding an *ASC1-birA** fusion gene, the *ASC1*-containing high-copy number plasmid pME2624 served as backbone. This plasmid was linearized by PCR with a forward primer annealing downstream of the *ASC1* ORF (5'- CCG CTC GAG TCA TGT AAT TA-3') and a reverse primer annealing to the 3'-end of the *ASC1* ORF excluding the stop codon (5'-AGT GGC ACG GTG TTA TCC TTA GAA GAA CCA GAA GAA CCA GAA GAA CCA GAA GAA CCG TTA GCA GTC ATA ACT TGC C-3'). The reverse primer featured a large overhang containing a 36 base pair linker sequence and a sequence complementary to the first 20 base pairs of the *birA** gene after the start codon. The *birA** allele containing the point mutation R118G was amplified from plasmid pRS313 without its start codon (fw: 5'- AAG GAT AAC ACC GTG CCA CT-3'; rv: 5'- ATT ACA TGA CTC GAG TTA TTT TTC TGC ACT ACG CA-3'). The reverse primer contained a sequence complementary to the plasmid backbone. The linearized plasmid backbone and the *birA** fragment were fused by homologous recombination using the *In-Fusion® HD Cloning Kit* (#639650, Clontech, Mountain View, California, USA). The coding sequence of the *ASC1-birA** fusion was verified by DNA sequencing and the plasmid was named pME4478. Similarly, a plasmid expressing the mere *birA** was constructed: Plasmid pME2624 was linearized by PCR with the forward primer annealing downstream of the *ASC1* ORF (5'- CCG CTC GAG TCA TGT AAT TA-3') and a reverse primer annealing to the plasmid backbone upstream of the *ASC1* ORF with a 20 bp overhang complementary to the *birA** gene (5'- GGT GTT ATC CTT CAT GAA TTC CTG CAG CCC GGG GG-3'). The *birA** allele including the ATG start codon was amplified from plasmid pRS313 (fw: 5'- ATG AAG GAT AAC ACC GTG CCA CT-3'; rv: 5'- ATT ACA TGA CTC GAG TTA TTT TTC TGC ACT ACG CA-3') and fused with the linearized plasmid backbone by homologous recombination. The coding sequence of *birA** was verified by DNA sequencing and the plasmid was named pME4480. A plasmid encoding an *asc1*^{DE}-*birA** fusion was generated by site directed mutagenesis. The mutated *asc1*^{DE} allele features two amino acid exchanges: R38D and K40E. To insert these substitutions within the *ASC1-birA** allele, pME4478 bearing the *ASC1-birA** fusion gene was used as template. With a complementary primer pair carrying the two mutations in its central part (fw: 5'-GTT GTC CGC TTC CGA TGA TGA AAC TTT GAT CTC CTG G-

3'; rv: CCA GGA GAT CAA AGT TTC ATC ATC GGA AGC GGA CAA C-3'), the *ASC1-birA** plasmid was fully amplified resulting in the *asc1*^{DE}*-birA** vector. The template DNA was removed by *Dpn*I treatment which exclusively digests methylated DNA, and thus only the parental vectors. The resulting plasmid was verified by DNA sequencing and termed pME4479.

Supplemental Figures

Figure S1: Overall protein biotinylation at 30°C vs. 37°C. Prior to combining cells of the three strains/conditions of the "heat" BioID experiment and before biotin affinity capture an aliquot from each culture was taken to compare the level of biotinylation. Biotinylated proteins were visualized with Streptavidin-HRP after Western blotting. The level of biotinylation was normalized according to the Ponceau-stained proteins of the whole proteome. The relative intensities (rel.int.) are indicated with standard deviation (SD).

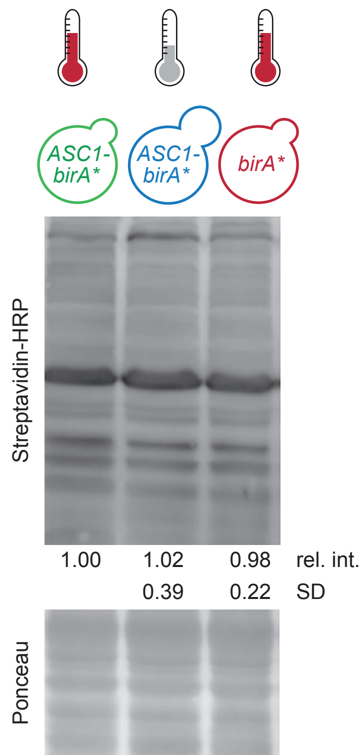

Figure S1

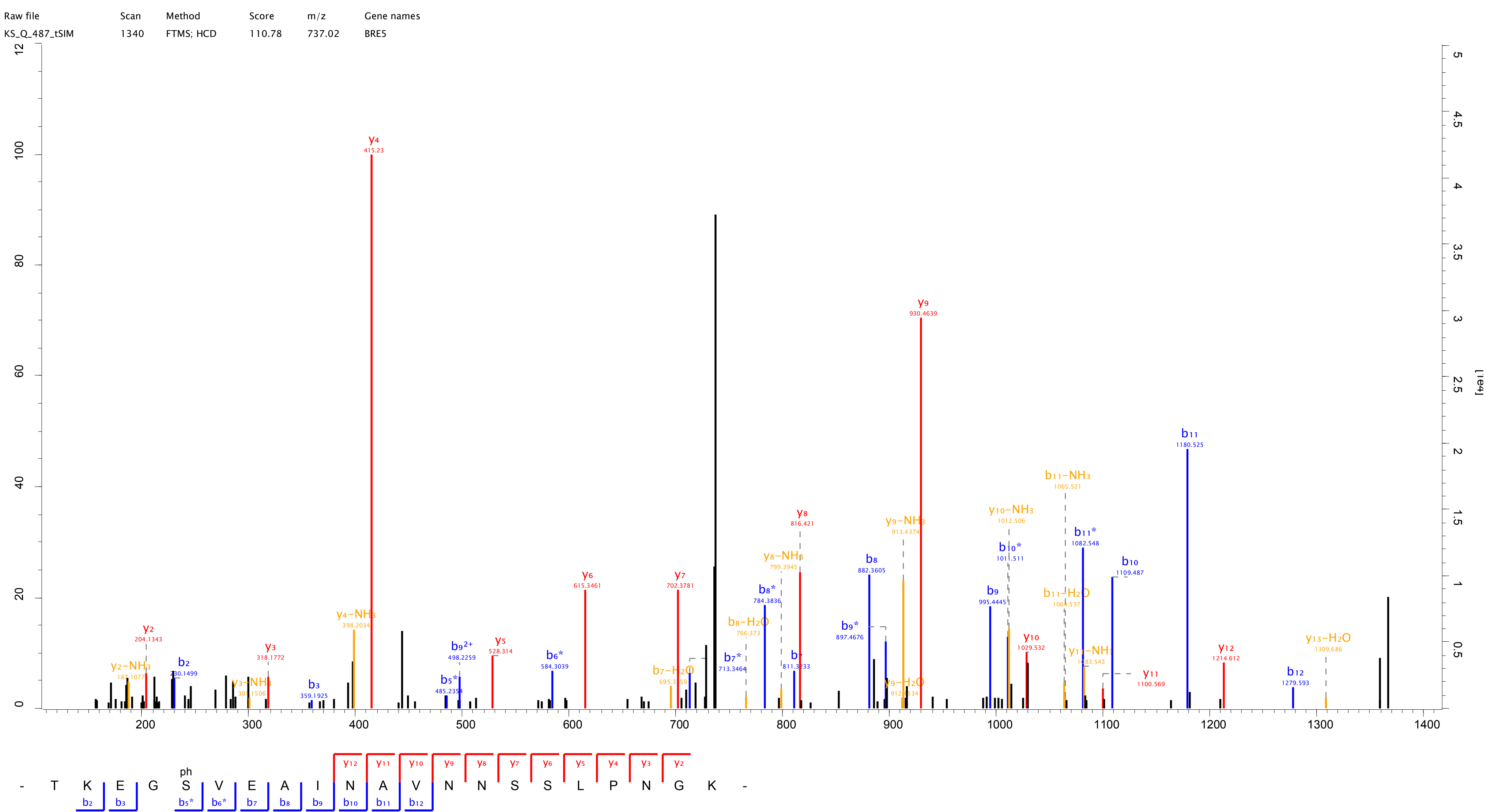

Figure S2: Representative fragmentation spectrum of the phosphorylated Bre5p peptide TKEGSVEAINAVNNSSLPNGK annotated with MaxQuant.

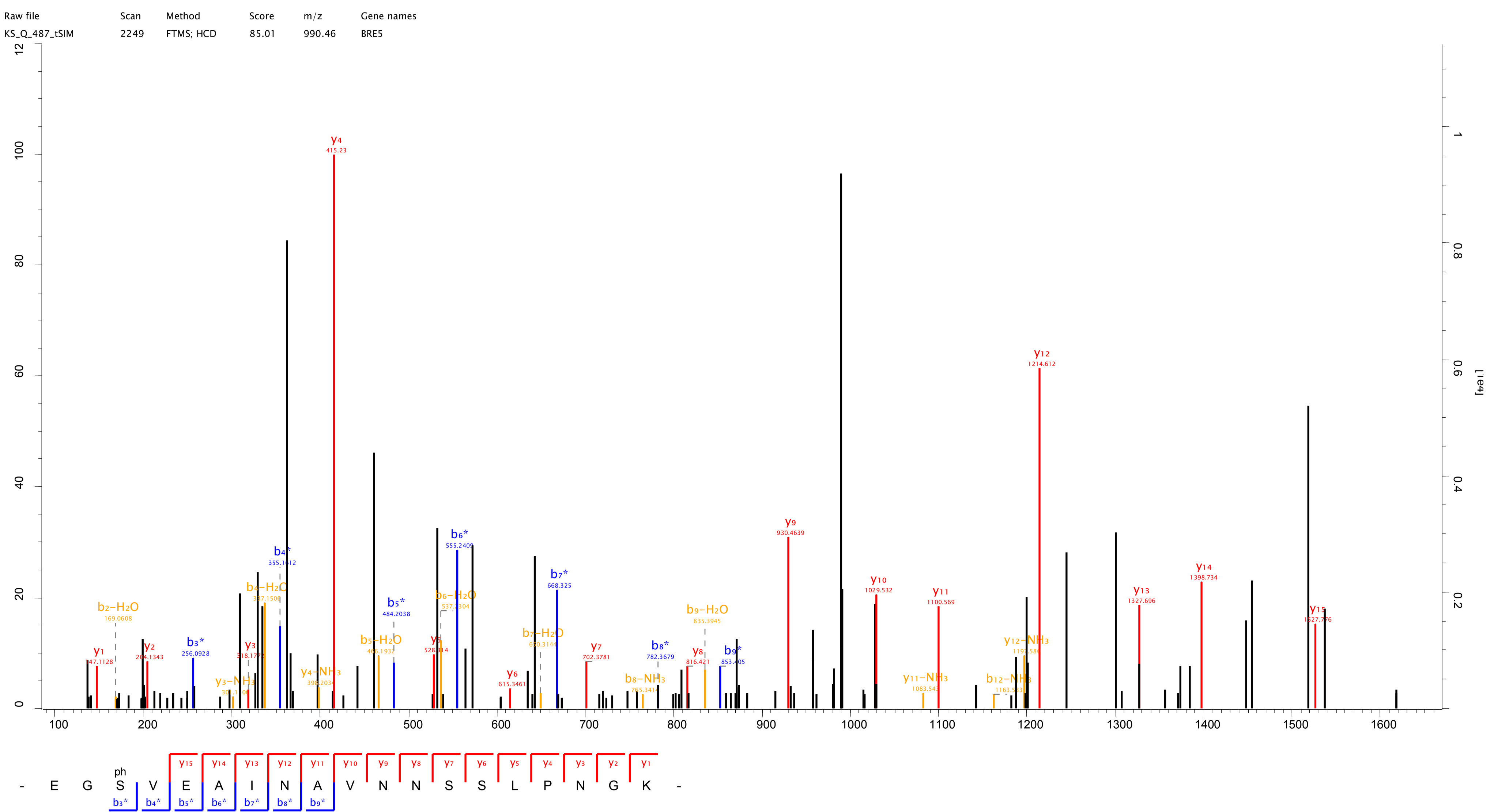

Figure S3: Representative fragmentation spectrum of the phosphorylated Bre5p peptide EGSVEAINAVNNSSLPNGK annotated with MaxQuant.