1 **Supplementary Materials and Methods**

2 **TABLE S1. Plasmids used in this work**

Plasmid	Description	Reference
pUG72	*Amp^R*, *pUCori*, *loxP::URA3::loxP*	25
pSH63	*Amp^R*, *pUCori*, *GAL1Prom*, *cre*, *TRP1*, *CEN/ARS*	25
pME2791	pRS416GAL1 with *GAL1Prom CYC1Term*, *URA3*, *CEN/ARS*	75
pME2787	pRS426MET25 with *MET25*Prom. *CYC1Term*, *URA3*, 2µm	75
pME2624	*MET25Prom*, *CYC1Term*, *URA3*, 2 µm, *ASC1*	our collection
pME2532	*MET25Prom*, *CYC1Term*, *URA3*, 2 µm, *Strep for C-terminal fusion*	our collection
pME2536	*MET25Prom*, *CYC1Term*, *URA3*, 2 µm, *Strep for N-terminal fusion*	our collection
pME2834	*MET25Prom*, *CYC1Term*, *URA3*, 2 µm, *ASC1-Strep*	our collection
pME2835	*MET25Prom*, *CYC1Term*, *URA3*, 2 µm, *Strep-ASC1*	our collection
pASK-IBA7plus	*AmpR*, *f1 origin, Strep*-tag, Xa cleavage site	IBA GmbH
pME4132	*AmpR*, *f1 origin, Strep-Xa-ASC1*	This work
pME4135	*MET25Prom*, *CYC1Term*, *bla*, *URA3*, 2 µm, *Strep-Xa-ASC1*	This work
pME4364	pME2791 with *ASC1* and its native promoter (+500bp)	This work
pME4384	pME2791 with asc*1*R38DK40E (*asc1*DE) and its native promoter	
pME4124	pME2834 with *asc1*DE	This work
pME4386	pME4364 with *asc1*DE-D109Y	This work
pME4365	pME4364 with *asc1*^{T12A}	This work
pME4366	pME4364 with *asc1*^{T12A}DE	This work
pME4367	pME4364 with *asc1*^{T12E}	This work
pME4368	pME4364 with *asc1*^{T12E}DE	This work
pME4025	pME2834 with *asc1*^{T96A}	This work
pME4370	pME2834 with *asc1*^{T96A}DE	This work
pME4026	pME2834 with *asc1*^{T96E}	This work
pME4371	pME2834 with *asc1*^{T96E}DE	This work
pME4027	pME2834 with *asc1*^{T99A}	This work
pME4372	pME2834 with *asc1*^{T99A}DE	This work

pME4028	pME2834 with *asc1*^{T99E}	This work
pME4373	pME2834 with *asc1*^{T99E}	This work
pME4029	pME2834 with *asc1*$^{T96A\ T99A}$	This work
pME4125	pME2834 with *asc1*$^{T96A\ T99A}$DE	This work
pME4030	pME2834 with *asc1*$^{T96E\ T99E}$	This work
pME4374	pME2834 with *asc1*$^{T96E\ T99E}$DE	This work
pME4385	pME4364 with *asc1*D109Y	This work
pME4120	pME2834 with *asc1*^{S120A}	This work
pME4375	pME4364 with *asc1*^{S120A}DE	This work
pME4121	pME2834 with *asc1*^{S120E}	This work
pME4122	pME2834 with *asc1*^{T143A}	This work
pME4395	pME4364 with *asc1*^{T143A}	This work
pME4376	pME2834 with *asc1*^{T143A}DE	This work
pME4396	pME4364 with *asc1*^{T143A}DE	This work
pME4473	pME4364 with *asc1*^{T143A}D109Y	This work
pME4123	pME2834 with *asc1*^{T143E}	This work
pME4387	pME4364 with *asc1*^{T143E}	This work
pME4377	pME2834 with *asc1*^{T143E}DE	This work
pME4388	pME4364 with *asc1*^{T143E}DE	This work
pME4474	pME4364 with *asc1*^{T143E}D109Y	This work
pME4031	pME2834 with *asc1*^{S166A}	This work
pME4126	pME2834 with *asc1*^{S166A}DE	This work
pME4032	pME2834 with *asc1*^{S166E}	This work
pME4033	pME2834 with *asc1*^{T168A}	This work
pME4127	pME2834 with *asc1*^{T168A}DE	This work
pME4034	pME2834 with *asc1*^{T168E}	This work
pME4035	pME2834 with *asc1*$^{S166A\ T168A}$	This work
pME4128	pME2834 with *asc1*$^{S166A\ T168A}$DE	This work
pME4036	pME2834 with *asc1*$^{S166E\ T168E}$	This work
pME4378	pME2834 with *asc1*^{Y250F}	This work
pME4379	pME2834 with *asc1*^{Y250F}DE	This work
pHK697	*RPS2-GFP*, *URA3*, *CEN/ARS*	76

TABLE S2. ***S. cerevisiae*** **strains used in this work**

For Asc1p phospho-site mutations that caused Asc1p-dependent phenotypes at least two independent clones with individual strain designations were generated and tested. The only exception is the *asc1*$^{\text{T12A}}$DE strain.

Strains	Genotype	Reference
RH2817	*MATα*, *ura3-52*, *trp1::hisG*	19
RH3263	*MATα*, *ura3-52*, *trp1::hisG*, *leu2::hisG*, Δ*asc1::LEU2*	19
RH3510	*MATα*, *ura3-52*, *trp1::hisG*, *asc1-loxP SNR24*	15
Y02724	BY4741, Mat a, *his3Δ1*, Δ*leu2*, Δ*met15*, Δ*ura3*, *YLR113w(HOG1)::kanMX4*	Euroscarf collection
RH3500	*MATα*, *ura3-52*, *trp1::hisG*, Δ*asc1::URA3*	This work
RH3549, RH3550	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*DE	This work
RH3431	*MATα*, *ura3-52*, *trp1::hisG*, *FLO8*-myc^{3}-*TRP1*	15
RH3504	*MATα*, *ura3-52*, *trp1::hisG*, *asc1-loxP SNR24*, *FLO8*-myc^{3}-*TRP1*	15
RH3599	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*DE, *FLO8*-myc^{3}-*TRP1*	This work
RH3623, RH3624, RH3625	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T12A}}$	This work
RH3626	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T12A}}$DE	This work
RH3627, RH3628, RH3629	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T12E}}$	This work
RH3630, RH3631	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T12E}}$DE	This work
RH3529	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T96A}}$	This work
RH3539, RH3540, RH3541	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T96A}}$DE	This work
RH3530	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T96E}}$	This work
RH3542, RH3543	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T96E}}$DE	This work
RH3531	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T99A}}$	This work
RH3611, RH3612, RH3613	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T99A}}$DE	This work
RH3532	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T99E}}$	This work
RH3614, RH3615, RH3616	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T99E}}$DE	This work
RH3533, RH3534, RH3535	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{\text{T96A T99A}}$	This work

RH3544, RH3545, RH3546	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{T96A\ T99A}$DE	This work
RH3537, RH3538	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{T96E\ T99E}$	This work
RH3547, RH3548	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{T96E\ T99E}$DE	This work
RH3551	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{S120A}	This work
RH3575, RH3576, RH3577	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{S120A}DE	This work
RH3574	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{S120E}	This work
RH3578, RH3579, RH3580	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T143A}	This work
RH3584, RH3585, RH3586	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T143A}DE	This work
RH3581, RH3582, RH3583	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T143E}	This work
RH3587, RH3588, RH3589	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T143E}DE	This work
RH3590	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{S166A}	This work
RH3638	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{S166A}DE	This work
RH3591, RH3592	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{S166E}	This work
RH3593, RH3594, RH3595	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T168A}	This work
RH3639, RH3640	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T168A}DE	This work
RH3596, RH3597	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{T168E}	This work
RH3598	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{S166A\ T168A}$	This work
RH3536	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{S166A\ T168A}$DE	This work
RH3637	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*$^{S166E\ T168E}$	This work
RH3635, RH3641, RH3642	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{Y250F}	This work
RH3636, RH3643, RH3644	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*^{Y250F}DE	This work
RH3487	*MATα*, *ura3-52*, *trp1::hisG*, Δ*arg4::URA3*	This work
RH3489	*MATα*, *ura3-52*, *trp1::hisG*, Δ*arg4::loxP*	This work
RH3491	*MATα*, *ura3-52*, *trp1::hisG*, Δ*arg4::loxP*, Δ*lys1::URA3*	This work
RH3493	*MATα*, *ura3-52*, *trp1::hisG*, Δ*arg4::loxP*, Δ*lys1::loxP*	This work
RH3519	*MATα*, *ura3-52*, *trp1::hisG, asc1-URA3 SNR24*, Δ*arg4::loxP*, Δ*lys1::loxP*	This work
RH3520	*MATα*, *ura3-52*, *trp1::hisG*, *asc1-loxP SNR24* Δ*arg4::loxP*, Δ*lys1::loxP*,	This work
RH3570	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*DE, Δ*arg4::URA3*	This work

RH3571	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*DE, Δ*arg4::loxP*	This work
RH3572	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*DE, Δ*arg4::loxP*, Δ*lys1::URA3*	This work
RH3573	*MATα*, *ura3-52*, *trp1::hisG*, *asc1*DE, Δ*arg4::loxP*, Δ*lys1::loxP*	This work

Plasmid Construction

Plasmids used in this study are listed within Table S1. Plasmids pME2834 and pME2835 were generated through amplification of *ASC1* from the yeast genome using oligonucleotides that introduced *Bam*HI and *Hind*III restriction sites for cloning into plasmids pME2535 and pME2536 respectively. To generate plasmid pME4135, *Strep-ASC1* was amplified from plasmid pME4132 introducing *Nhe*I and *Hind*III restriction sites for cloning into plasmid pME2835. Plasmid pME4132 was obtained through insertion of the *ASC1* gene into the pASK-IBA7plus plasmid (#2-1406-000, IBA GmbH) according to the provided instructions. Plasmid pME4364 is derived from pME2791 and carries *ASC1* under control of its native promoter (+500 bp upstream of *ASC1* according to (77). *ASC1* was amplified together with its promoter from genomic *S. cerevisiae* DNA using oligonucleotides that introduced *Sac*I and *Hind*III restriction sites for subsequent cloning. Plasmids carrying *asc1* with codon exchanges were constructed via a two-step PCR strategy: In the first reaction the codon exchange(s) was (or were) introduced within an oligonucleotide bearing the mutated codon(s) in its center using an *ASC1* wild-type carrying plasmid (pME2834 or pME4364) as template. In the second PCR the complete *asc1* allele was amplified flanked by the respective restriction sites for cloning into the parent vector (*Bam*HI and *Hind*III restriction sites for cloning into pME2834 and *Sac*I and *Hind*III restriction sites for cloning into pME2791). For construction of plasmids pME4025-pME4036, pME4120-pME4128, pME4370-pME4374, and pME4376-pME4379 plasmid pME2834 served as the parent vector. Plasmid pME4364 served as

28 parent vector for plasmids pME4365-pME4368, pME4375, and pME4384. For plasmids
29 comprising an R38D K40E codon exchange in combination with a phosphorylation site mutation
30 either plasmid pME4124 or pME4384 was used as template for PCR. Alternatively, plasmids that
31 already comprised a phosphorylation site mutation were used as template for the PCR and the
32 R38D K40E mutation was introduced by using oligonucleotides bearing the respective codon
33 exchanges. The plasmids served as templates for amplification of transformation cassettes to
34 integrate the mutated *asc1* alleles into the yeast genome at its original locus.

35

36 *Yeast strains and growth conditions*

37 The *S. cerevisiae* strains used in this study are of the Σ1278b background and are listed Table S2.
38 Strains with codon-exchanges within the open-reading frame of *ASC1* (RH3529-RH3551,
39 RH3574-RH3598, RH3611-RH3616, RH3623-RH3631, RH3635-RH3644) were obtained by
40 transformation of the Δ*asc1::URA3* strain (RH3500) with the respective *asc1*-alleles carrying
41 flanking regions identical to those of the *ASC1* gene for homologues recombination. Transformants
42 were selected for their resistance against 5-fluoroorotic acid (0.5 mg/ml; #R0812, Thermo Fisher
43 Scientific) due to loss of the *URA3* marker gene. Successful transformations were verified by PCR,
44 Southern blot (78), and sequencing of the *ASC1* locus. Arginine or arginine and lysine auxotrophic
45 strains RH3487, RH3489, RH3491, RH3493, and RH3570-RH3573 derived from strains RH2817
46 and RH3549, respectively, and were generated by replacement of the genes *ARG4* and *LYS1* with
47 the recyclable *loxP::URA3::loxP* marker cassette that was amplified from plasmid pUG72
48 according to Gueldener et al. (25). For marker rescue strains were transiently transformed with
49 plasmid-borne Cre-recombinase (pSH63). An arginine and lysine auxotrophic *asc1SNR24* strain
50 was generated from the *ASC1* wild-type Δ*arg4::loxP* Δ*lys1::loxP* strain RH3493 as described by
51 Rachfall et al. (15): A *loxP::URA3::loxP* cassette was integrated in exon1 of *ASC1* resulting in

strain RH3519. Subsequent rescue of the *URA3* marker led to strain RH3520 with a *loxP* site in the *ASC1* ORF that abrogates the translation of the mRNA. For metabolic labeling with isotopically-labeled arginine and lysine Δ*arg4::loxP* Δ*lys1::loxP* strains RH3493, RH3520, and RH3573 were used. Strain RH3599 expressing C-terminally 3xmyc-tagged Flo8p was constructed by transformation of RH3549 according to Janke et al. (26). Transformations were performed using the lithium acetate method (79). Strains were cultivated in liquid yeast nitrogen base medium (1.5 g/l YNB without amino acids and ammonium sulfate, 5 g/l ammonium sulfate, 2% glucose) containing the respective supplements or in yeast-extract peptone dextrose (YEPD) medium (2% peptone, 1% yeast extract, 2% glucose). 2% agar was added for solid media. Experiment-specific growth conditions are described in the respective paragraphs.

Supplementary References

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TABLE S3. LC-MS-based identification of Asc1p-derived phospho-peptides

Phospho-sites with the highest post-translational modification (PTM) score are printed in black, and all other putative phospho-sites within the peptides in gray.

Amino acid	Position	Highest PTM score	Peptide sequence	Spectral counts	Higest Xcorr (SequestHT)	Highest IonScore (Mascot)
T	12	100.00	G**T**LEGHNGWVTSLATSAGQPN LLLSASR	19	5.42	70
T	21	45.5				
S	22	33.3				
T	25	60.1				
S	26	39				
S	35	9.9				
S	37	79.6				
S	120	100	KA**S**MIISGSR	2	-	47
S	124	0				
S	126	0				
T	143	100	GQCLA**T**LLGHNDWVSQVR	29	4.54	59
S	152	0				
S	166	100	ADDD**S**V**T**IISAGNDK VVPNEKADDD**S**V**T**IISAGNDK	121 25	4.87 4.37	108 60
T	168	100				
S	171	90				
Y	250	100	**Y**WLAAATATGIK	21	3.51	56
T	256	0.3				
T	258	0				
S	291	77.4	AAEPHAVSLAWSADGQ**T**LFAG YTDNVIR	17	5.19	91
S	295	93.2				
T	300	99.9				
Y	305	0.6				
T	306	0.2				

TABLE S4. Overview on proteome data evaluation with *Perseus*

Abbreviations: Prot = protein, vs. = versus

No.	Command	Description	
		asc1$^-$ and *asc1*DE	T143 phosphorylation-site mutant strains e.g. *asc1*^{T143A}
1	Generic matrix upload	proteinGroups.txt normalized ratios etc.	
2.1 2.2 2.3	Filter rows based on categorical columns	Remove rows with + in reverse column Remove rows with + in potential contaminant column Remove rows with + in only identified by site	
3	Transform	Inverse ratios (1/x) when *ASC1* is not in the denominator	
4	Transform	$log_2(x)$	
5	Normali-zation	Subtract column median of ratios	
6	Categorical annotation rows	Group biological replicates	
7	Reorder/ remove columns	Select Prot ratios of interest for the following steps	
8.1	Average groups	Calculate median of each group → *protein ratio*	Calculate mean of each group
8.2	Combine expression columns	-	Calculation of difference between mean of *asc1*^{T143A}/*ASC1* ratios and *ASC1*^{Aux}/*ASC1* ratios → *protein ratio*
9	Change column type	Change numerical column with *protein ratio* (steps 8.1 and 8.2) to expression column	
10	Categorical annotation rows	Define column with protein ratio as own group	
11	One/Two sample(s) tests	One sample t-test, p value 0.01	Two sample t-test, p value 0.01 Prot ratios *asc1*^{T143A}/*ASC1* vs. Prot ratios *asc1*^{Aux}/*ASC1*
12	Filter rows based on categorical columns	Keep rows with + (significant) from step 11	
13	Filter rows based on valid values	Filter *protein ratio* (steps 8.1 and 8.2) for values outside -0.26 to 0.26	

TABLE S5. Overview on phospho-proteome data evaluation with *Perseus*

Abbreviations: PP = phospho-peptide, Prot = protein, norm. = normalized, vs. = versus

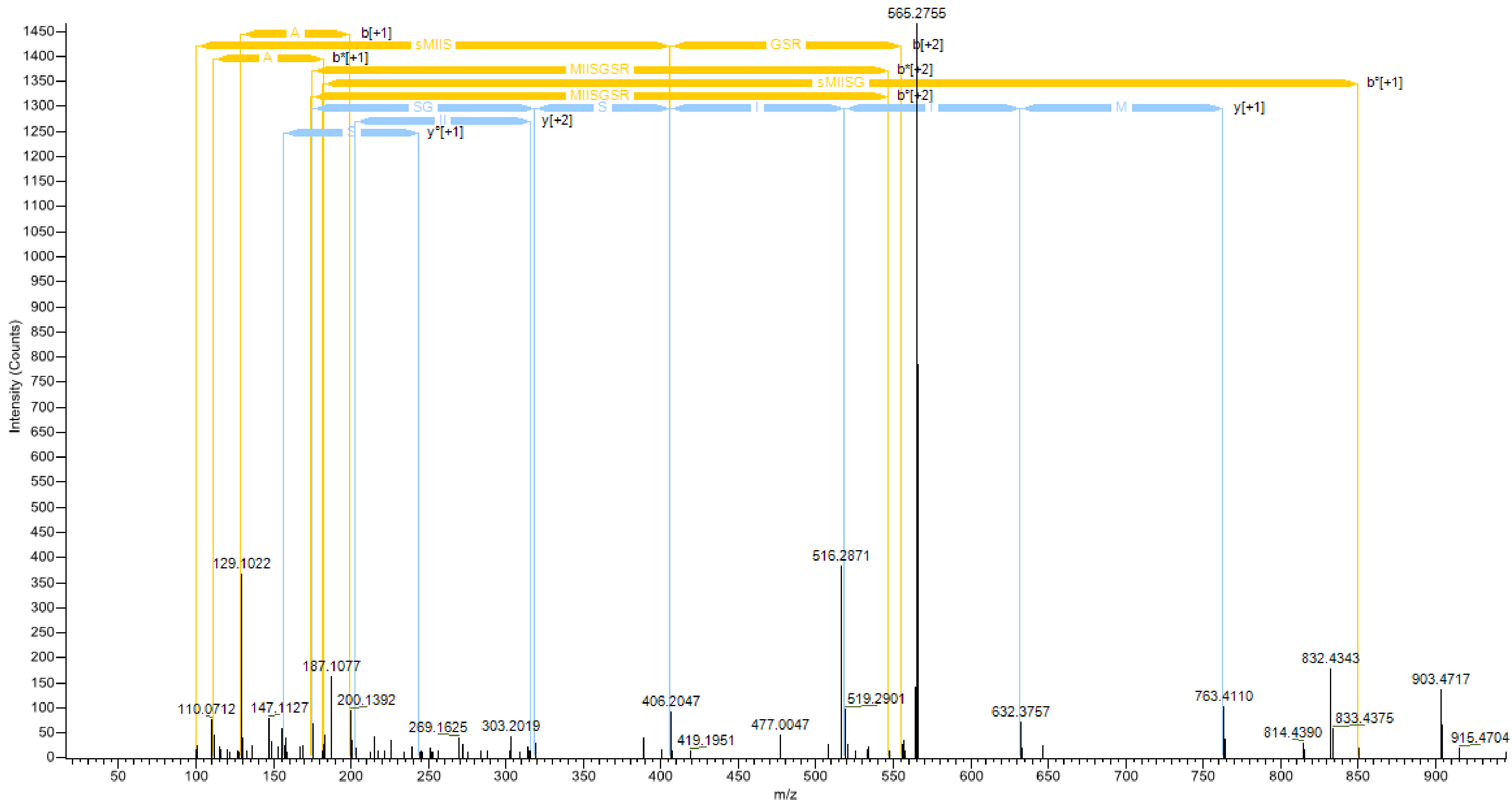
No.	Command	Description	
		asc1^{-}	T143 phosphorylation-site mutant strains e.g. *asc1*^{T143A}
1	Generic matrix upload	Phospho (STY)sites.txt normalized ratios, localization probability etc.	
2.1 2.2	Filter rows based on categorical columns	Remove rows with + in reverse column Remove rows with + in potential contaminant column	
3	Expand site table	In order to have only one column per sample	
4	Transform	Inverse ratios (1/x) when *ASC1* is not already in the denominator	
5	Transform	$log_2(x)$	
6	Reorder/ remove columns	Select PP ratios of interest for the following steps	
7	Matching rows by name	Matching PP ratios with respective Prot ratios from step 7 of Table S2 (in the case that a phospho-peptide cannot be assigned to a single protein the median of the protein ratios is calculated)	
8	Categorical annotation rows	Group PP ratios and Prot ratios for biological replicates	
9	Average groups	Calculate median of each group	Calculate mean of each group
10.1	Combine expression columns	Normalization of PP ratios on Prot ratios: median PP ratios - median Prot ratios → *phospho regulation*	
10.2		-	Calculation of difference between normalized PP ratios of *asc1*^{T143A}/*ASC1* and *ASC1*^{Aux}/*ASC1* → *phospho regulation*
11	Change column type	Change numerical column with *phospho regulation* (steps 10.1 and 10.2) to expression column	
12	Categorical annotation rows	Define column with *phospho regulation* as an own group for later filtering of values	
13	Categorical annotation rows	Group PP ratios for biological replicates for the next step	
14	Filter rows based on valid values	Filter for two valid PP ratios	Filter for one valid PP ratio
15.1	Two samples tests	t-test, p value 0.01 PP ratios vs. Prot ratios	t-test, p value 0.05 PP ratios vs. Prot ratios for *asc1*^{T143A}/*ASC1*
15.2		-	t-test, p value 0.05 PP ratios *asc1*^{T143A}/*ASC1* vs. PP ratios *ASC1*^{Aux}/*ASC1*
16.1	Filter rows	Keep rows with + (significant) from step 15.1	

16.2	based on categorical columns	-	Keep rows with + (significant) from step 15.2
17	Filter rows based on valid values	Filter *phospho regulation* (steps 10-12) for values outside -0.26 to 0.26	
Following steps only performed for *asc1*$^{-}$ phospho-proteome analysis for phosphorylated peptides with zero to two corresponding prot ratios Start from step 15.1			
18	One sample tests	t-test, p value 0.01 only phospho-peptide ratios	
19	Filter rows based on categorical columns	Discard rows with + (significant) from step 15.1 (these candidates showed regulation of PP ratios with respect to Prot values)	
20	Filter rows based on categorical columns	Keep rows with + (significant) from step 18	
21	Categorical annotation rows	Group Prot ratios → *proteome*	
22	Filter rows based on valid values	Filter rows on three valid values in the *proteome* group, add categorical column (assumption: less than three valid values might be not sufficient for two samples t-test)	
23	Filter rows based on categorical columns	Keep rows that were specified as *discarded* in the previous step (remaining candidates have less than three valid values in the *proteome* group)	
24	Filter rows based on valid values	Filter rows on one valid values in *proteome* group, add categorical column	
		phosphorylated peptides with no corresponding Prot ratio	phosphorylated peptides with one or two corresponding Prot ratio(s)
25	Filter rows based on categorical columns	Keep rows that were specified as *discarded* in the previous step, they have no *proteome* values	Keep rows that were specified as *keep* in the previous step, they have one or two *proteome* value(s)
26	Change column type	Change numerical column with PP median ratio (step 9.1) to expression column	-
27	Categorical annotation rows	Define column with PP median ratio from the previous step as an own group for filtering of values in the next step → *phospho regulation with no Prot value*	-
28	Filter rows based on valid values	Filter *phospho regulation with no Prot value* (step 27) for values outside -0.26 to 0.26	Filter *phospho regulation* (step 10.1) for values outside -0.26 to 0.26

1

FIG. S1. Annotated fragmentation spectra of Asc1p phospho-peptides.

(A) Annotated fragmentation spectrum of the peptide GTLEGHNGWVTSLATSAGQPNLLLSASR phosphorylated at T12



(B) Phosphorylated fragmentation spectrum of the peptide KASMIISGSR phosphorylated at S120

■ = fragment b-ions   * = neutral loss of ammonia (-17 Da)   [+1], [+2], [+3] = charge states

■ = fragment y-ions   ° = neutral loss of water (-18 Da)   lower case *t*/*c* = phosphorylated residue/carbamidomethylated cysteine

KS121_MSA_3.raw #7945 RT: 85.24

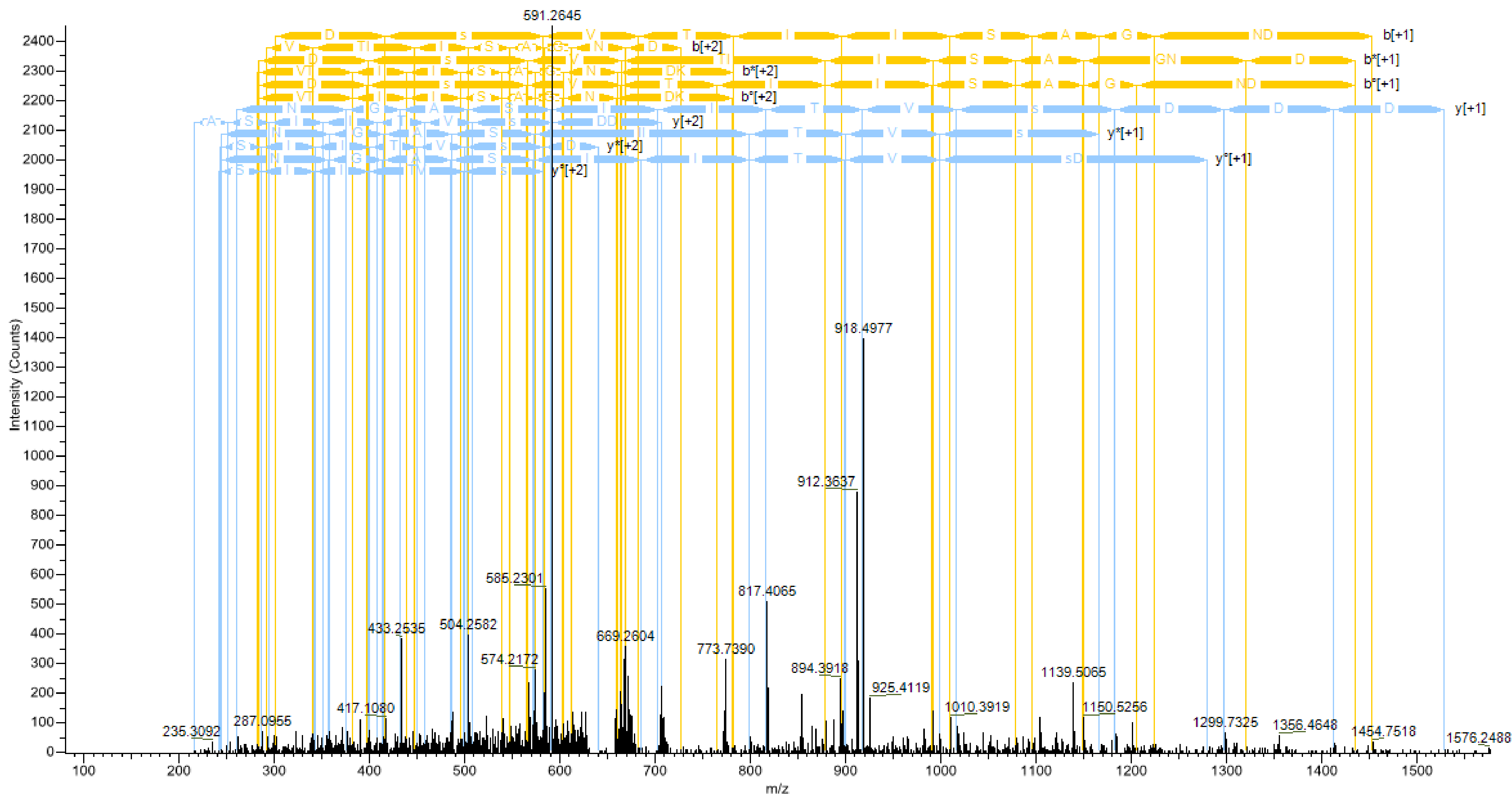
ITMS, CID, z=+3, Mono m/z=712.00018 Da, MH+=2133.98600 Da, Match Tol.=0.6 Da

Intensity (Counts)

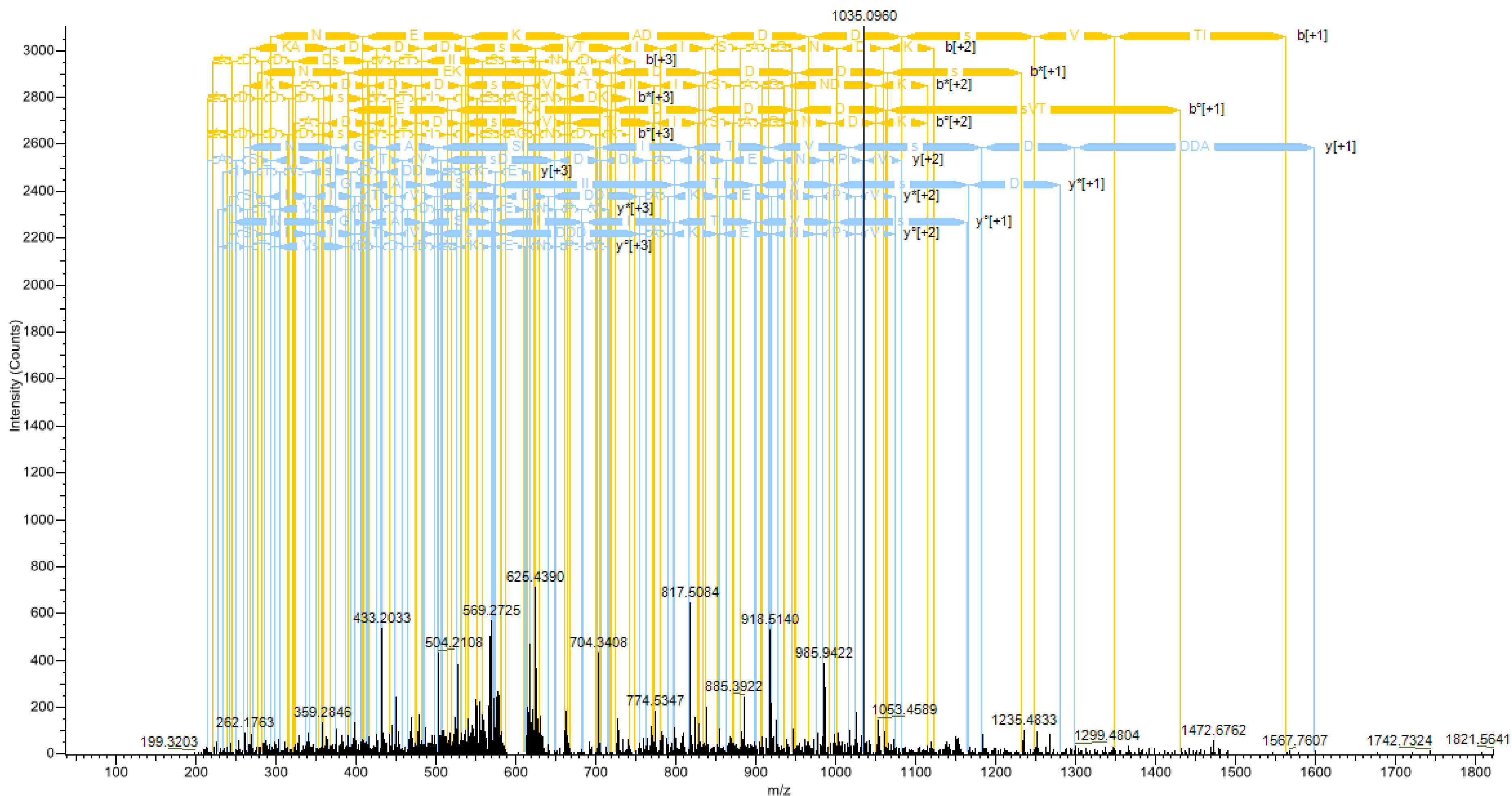
m/z

774.4099, 489.2744, 588.3092, 889.4261, 1048.6395, 581.8386, 570.7750, 1003.4505, 396.0330, 283.1426, 823.4620, 682.4224, 951.1140, 1197.5291, 402.3239, 274.1420, 373.9555, 230.0991, 1140.5493, 1249.6812, 1344.6959, 1520.7272, 1599.6461, 1769.6763, 1915.2323

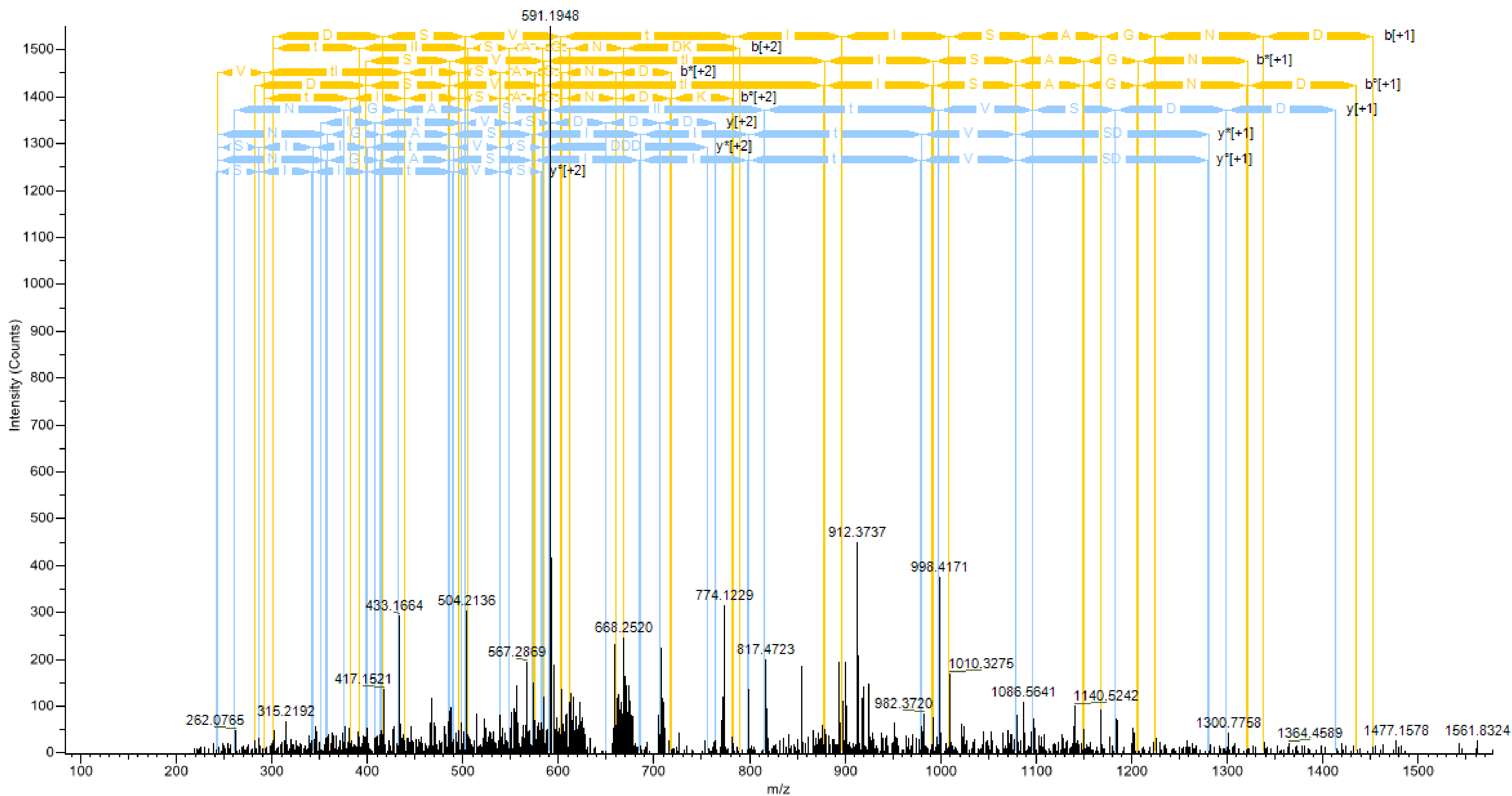
(C) Annotated fragmentation spectrum of the peptide GQCLATLLGHNDWVSQVR phosphorylated at T143



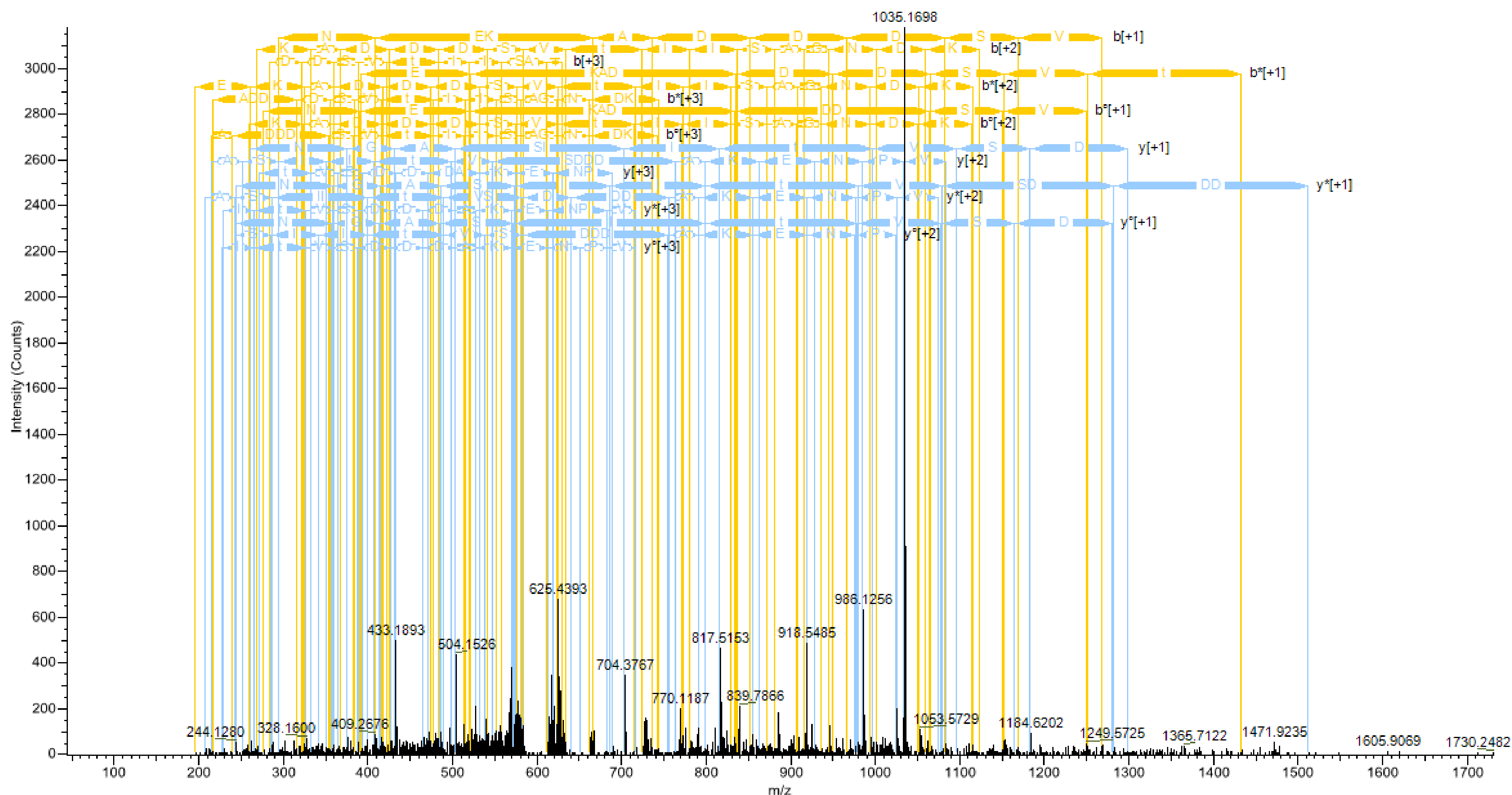
(D) Annotated fragmentation spectrum of the peptide ADDDSVTIISAGNDK phosphorylated at S166



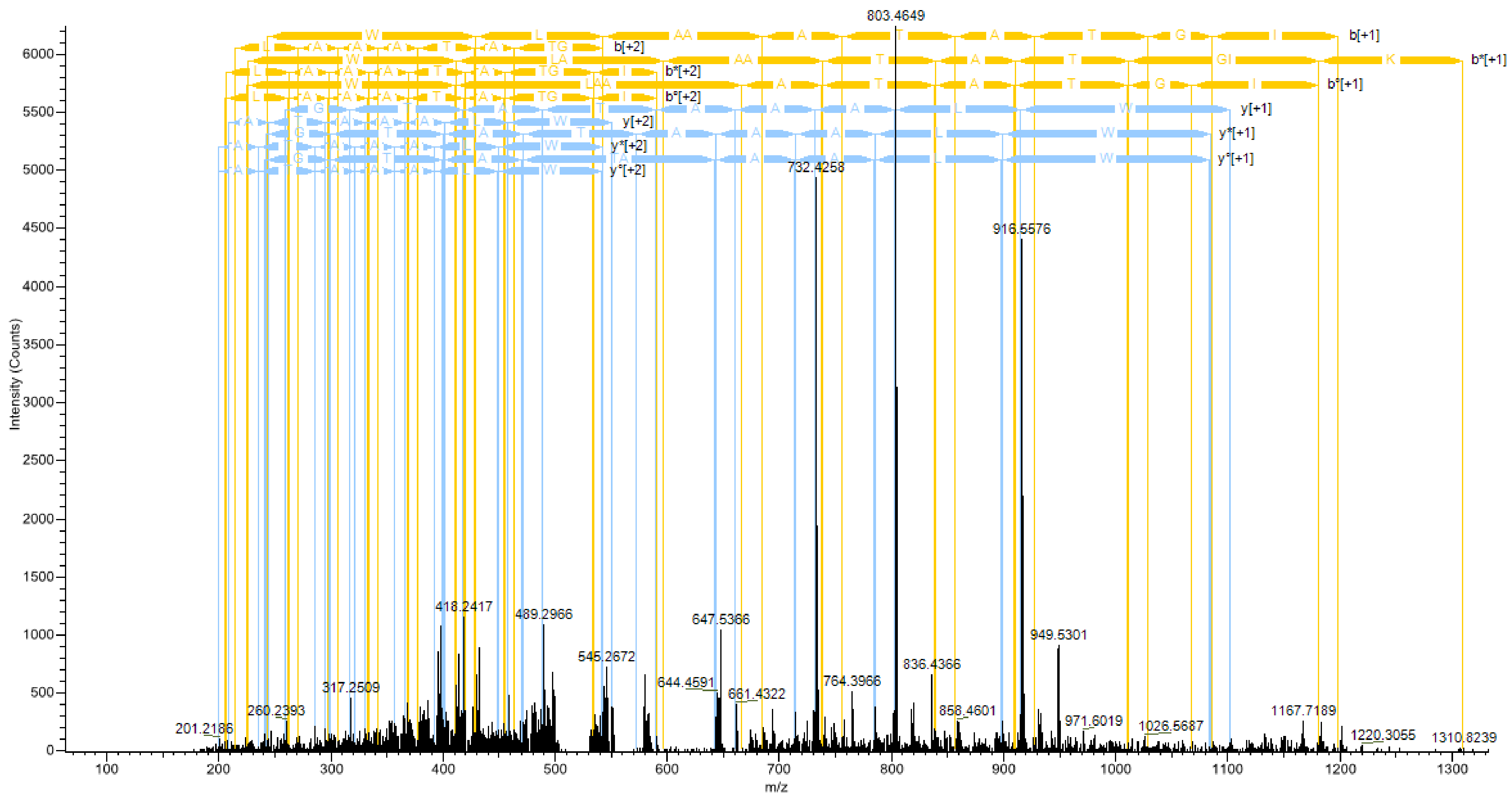
(E) Annotated fragmentation spectrum of the peptide VVPNEKADDDSVTIISAGNDK phosphorylated at S166



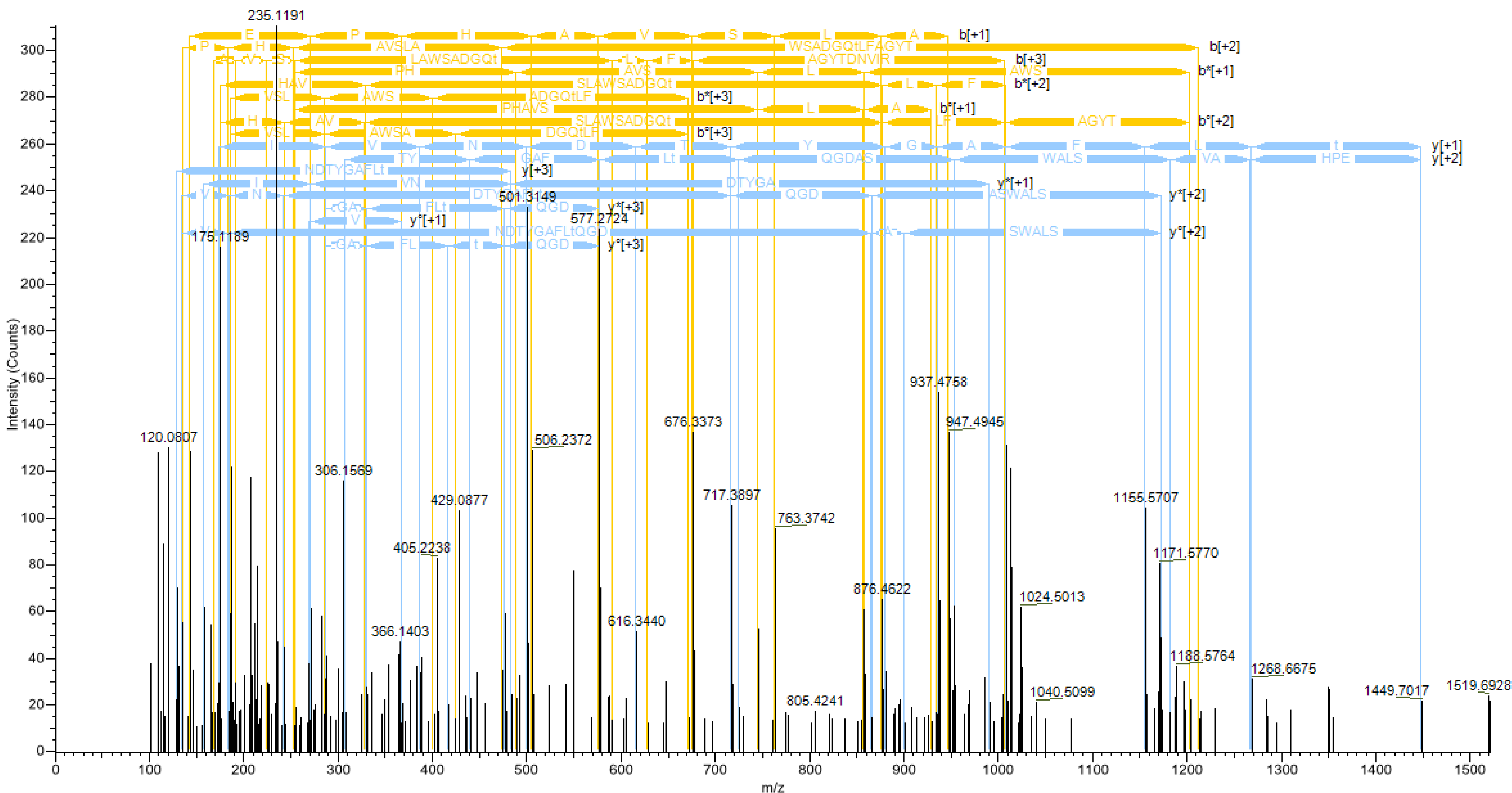
(F) Annotated fragmentation spectrum of the peptide ADDDSVTIISAGNDK phosphorylated at T168



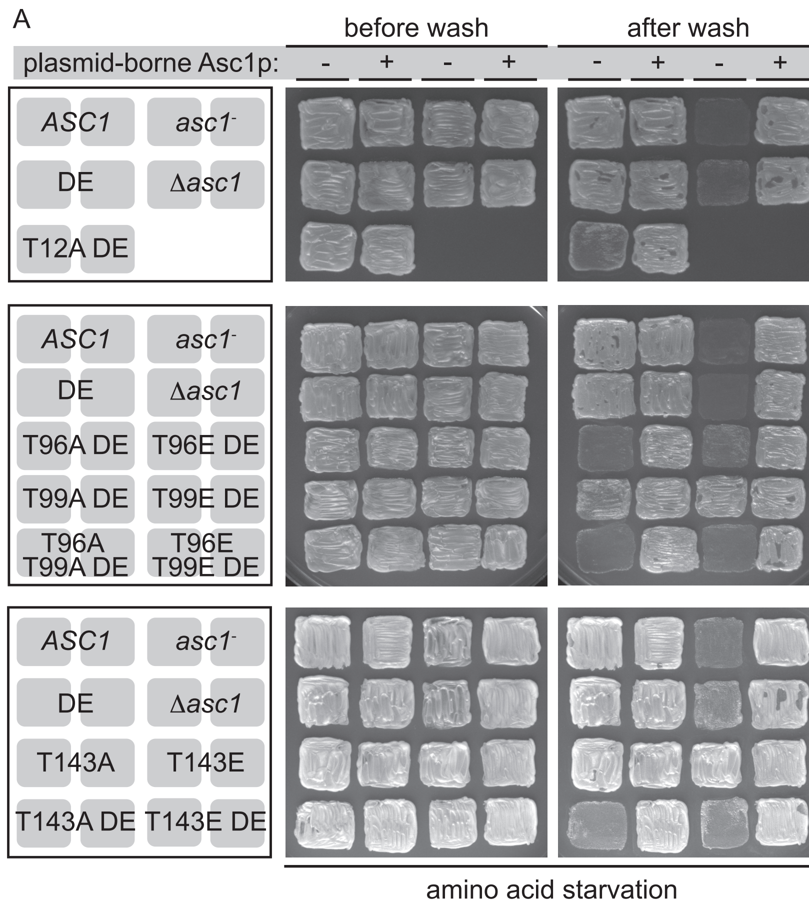
(G) Annotated fragmentation spectrum of the peptide VVPNEKADDDSVTIISAGNDK phosphorylated at T168



(H) Annotated fragmentation spectrum of the peptide YWLAAATATGIK phosphorylated at Y250



(I) Annotated fragmentation spectrum of the peptide AAEPHAVSLAWSADGQTLFAGYTDNVIR phosphorylated at T300



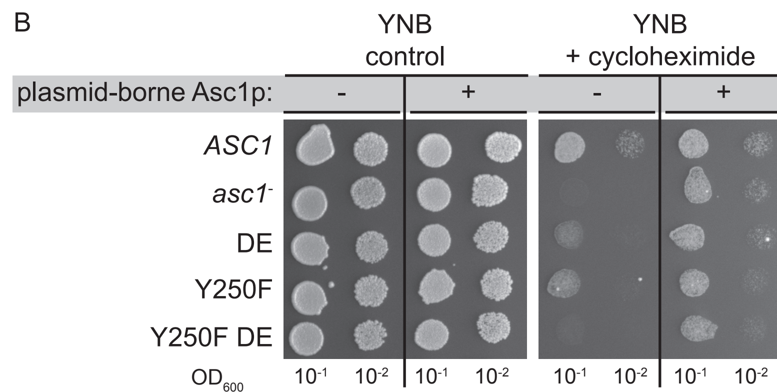


Figure S2

FIG. S2. Complementation of *ASC1* phospho-site mutant phenotypes by expression of plasmid-borne wild-type *ASC1*

Phospho-site mutants were transformed with a high-copy number plasmid carrying the wild-type *ASC1* gene under the control of the *MET25*-promoter (*ASC1*, pME2624) or with the empty vector (EV, pME2787) as control. The *ASC1* wild-type, *asc1*$^{-}$, Δ*asc1* and *asc1*DE strains were transformed with the same plasmids as further controls.

(A) Haploid adhesive growth at amino acid starvation caused by 10 mM 3-amino-1,2,4-triazole (3-AT). Plasmid-transformed phospho-site mutants *asc1*^{T12A}DE, *asc1*^{T96A}DE, *asc1*^{T96E}DE, *asc1*^{T99A}DE, *asc1*$^{T96A\ T99A}$DE, *asc1*$^{T96E\ T99E}$DE, *asc1*^{T143A}DE, and *asc1*^{T143E}DE and control strains were patched on the 3-AT containing YNB plates and subjected to wash tests after 3 d of growth. The *asc1*^{T99E}DE, *asc1*^{T143A}, and *asc1*^{T143E} strains, that are not impaired in adhesive growth, served as additional controls. Plates are shown before and after the washing step.

(B) Drop dilution assay to evaluate cycloheximide sensitivity. Cell suspensions of plasmid-transformed phospho-site mutant strains *asc1*^{Y250F} and *asc1*^{Y250F}DE and control strains were applied onto YNB plates containing 0.15 µg/ml.

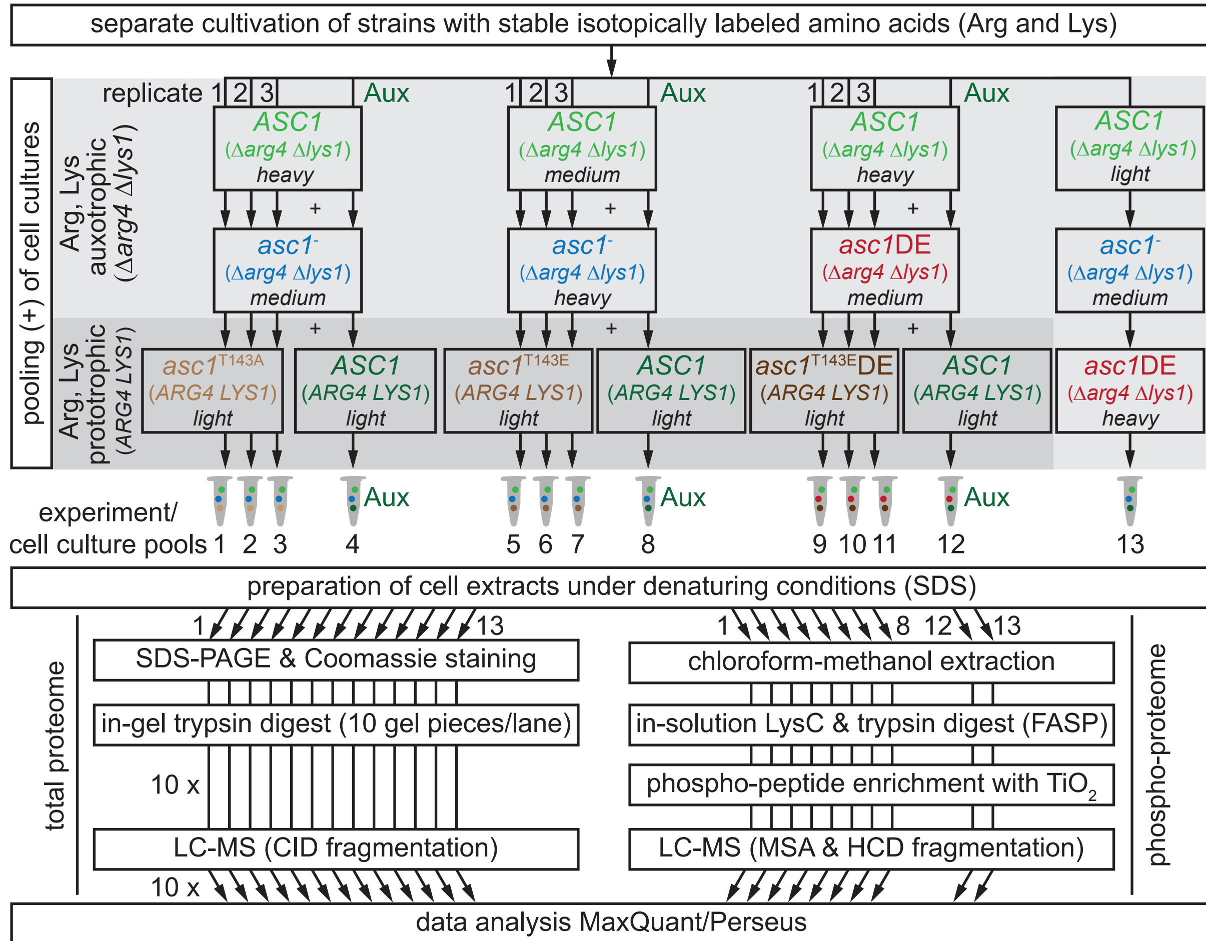


Figure S3

1 **FIG. S3. Analysis of the Asc1p-dependent phospho-proteome**

2 Peptide sample preparation for SILAC and LC-MS based Asc1p-dependent phospho-proteome
3 and proteome analyses. *S. cerevisiae* strains were cultivated in the presence of *light*, *medium* or
4 *heavy* labeled arginine and lysine. Arrows indicate which strains were pooled and indicate the
5 number of replicates. Strains with the phospho-site T143 mutated were arginine and lysine
6 prototroph and therefore cultivated in the presence of the naturally occurring light amino acids
7 (Arg^0, Lys^0). To distinguish between changes caused by the Asc1p mutations or differences in
8 arginine and lysine metabolism (prototroph *ARG4 LYS1* strains versus auxotroph Δ*arg4* Δ*lys1*
9 strains), an *auxotrophy-control ASC1*^{Aux} (Aux) was included in the experiments that is an *ASC1*
10 wild-type strain prototrophic for arginine and lysine (*ARG4 LYS1*). In total, 13 independent cell
11 pools were obtained and subjected to the subsequent preparation of cell extracts. The protein
12 extracts were split in two and processed separately for the proteome and phospho-proteome
13 analyses as described in detail in the *Materials and Methods* section. For the phospho-proteome
14 analysis only protein extracts of experiments 1-8 and 12-13 were further processed. The
15 encompassing set of data of the experiments and details about data analysis are compiled in the
16 Excel workbook Table S6 and Tables S4 and S5.