Supporting Online Material for

Stress-Inducible Regulation of Heat Shock Factor 1 by the Deacetylase SIRT1

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This PDF file includes

Supporting Online Material

Materials & Methods

Constructs, antibodies and reagents

mHSF1-Flag and hHSF1-Myc have been described earlier (1,2). HSF1 point mutants were created by QuikChange Site-directed mutagenesis (Stratagene) and confirmed by sequencing. For the Myc-HSF1 ten serine mutant, phosphorylated serine residues S230, S303, S307, S314, S319, S320, S338, S363, S368 and S369 (Ref. 3 and our unpublished data) were all mutated to alanines. SIRT1 WT and the H363Y mutant expression constructs were kindly provided by Dr. Tony Kouzarides (University of Cambridge) and the p300 expression construct from Dr. David Livingston (Harvard University). Antibodies used in this study are α-Flag M2 (Sigma), α-AcK (Cell Signaling 9441), α-HSF1 (4), α-SIR2 (Upstate Biotech #07-131), α-Myc (Clontech) and α–p300 (Santa Cruz). Chemical compounds used are nicotinamide (Sigma), trichostatin A (Upstate), EGS (Sigma), celastrol (GAIA Chemical Corporation), $CdCl_2$ (Sigma) and MG132 (Calbiochem).

Cell culture, transfections and treatment conditions

All cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere. K562 cells were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics (penicillin and streptomycin). 293T, HeLa, Cos7 and WI-38 cells were cultured in DMEM supplemented with 10% FCS and antibiotics. *hsf1*-/- mouse embryonic fibroblasts (5) were cultured in DMEM with 10% FCS, 10 mM non-essential amino acids, 0.96 µl of 2-mercaptoethanol per 100 ml, and antibiotics. 293T cells were transfected using Polyfect (Qiagen) according to the manufacturer's instructions. K562, Cos7 and *hsf1*-/- cells were transfected by electroporation with a Gene

Pulser electroporator (Bio-Rad). Heat shock was induced by submersion of cells in a prewarmed circulating water bath at 42°C for 1 hour. Celastrol treatment was at a concentration of 5 μM for 1 hour, $CdCl_2$ treatment was at 50 μM for 6 hours and MG132 was at 20 μM for 6 hours. Trichostatin A was added to cells overnight at 1 mM, nicotinamide overnight at 5 mM and resveratrol for 40 hours at 50 μM.

siRNA transfections

HeLa cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer's protocol, using 200 nM of Dharmacon SmartPool SIRT1 siRNA. Twenty-four hours later, cells were split and transfected again. RNA was isolated using Trizol 48 hours after the second transfection. Primers used to verify knockdown of SIRT1 were

(forward) 5'-TCCTGGACAATTCCAGCCATCTCT-3' and

(reverse) 5'-TTCCAGCGTGTCTATGTTCTGGGT-3'.

Acetylation assays

293T or Cos7 cells were transfected with Flag-HSF1 or Myc-HSF1 and p300, and then treated with or without nicotinamide prior to treatment with various stresses. Cell lysates were subjected to immunoprecipitation with a Flag antibody or Myc antibody, and acetylated HSF1 was detected by western blotting with an antibody that recognizes acetylated lysines.

Chromatin immunoprecipitation (ChIP)

ChIP reactions were performed essentially as previously described (6). Samples generated from HeLa cells ($3x10^7$) were immunoprecipitated with 10 µl α-HSF1 (4) at 4ºC overnight. Primers used for the hsp70.1 promoter (GenBank Acc# M11717) were (forward) 5'-GGCGAAACCCCTGGAATATTCCCGA-3' and (reverse) 5'-AGCCTTGGGACAACGGGAG-3' and surround the proximal HSE. Results were normalized to reactions performed with 1% of input.

Purification of HSF1 from 293T cells

293T cells were transfected with mHSF1-Flag with or without CMV-p300 as indicated with Polyfect (Qiagen) according to the manufacturer's protocol. Cells were treated with trichostatin A or nicotinamide as indicated 4 hours prior to performing stress treatments. Cell pellets were then harvested and lysed in RIPA buffer. mHSF1-Flag was immunoprecipitated with α-Flag M2 affinity gel beads (Sigma F2426) and eluted with Flag peptide. Samples were separated by SDS-PAGE prior to western blot analysis.

Mass spectrometry analysis

Purified mHSF1-Flag was obtained as described above from 293T cells transfected with mHSF1-Flag and CMV-p300 and treated with 1 µM trichostatin A and 5 mM nicotinamide for 18 hours prior to HS or celastrol treatment. Immunoprecipitated protein was separated by SDS-PAGE, excised from the gel, digested with trypsin, and subjected to tandem mass spectrometric analysis by a hybrid quadrupole time-of-flight instrument (QSTAR, Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray source. MS/MS spectra were searched against the IPI mouse sequence database (68,222 entries; version 3.15) using Mascot (Matrix Science, Boston, MA; version 1.9.05) and X! Tandem (www.thegpm.org; version 2006.04.01.2) database

search algorithms. Mascot and X! Tandem were searched with a fragment and precursor ion mass tolerance of 0.3 Da assuming the digestion enzyme trypsin with the possibility of one missed cleavage. Carbamidomethylation of cysteine was included as a fixed modification whereas methionine oxidation, N-terminal protein and lysine acetylation were included as variable modifications in the database search. Peptide identifications were accepted at greater than 95.0% probability as determined by the Peptide Prophet algorithm (7) and validated by manual inspection of the MS/MS spectra.

Recombinant proteins

Murine His-HSF1 was produced in the Escherichia coli strain BL-21 as previously described (8). Harvested bacteria were resuspended in cold lysis buffer [50 mM Tris-HCl (pH 7.4), 140 mM NaCl, 10% glycerol] and lysed with 1 mg/ml lysozyme at room temperature for 15 minutes. After addition of 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiotreitol) and EDTA-free protease inhibitor cocktail (Roche), lysates were sonicated and incubated with Talon metal affinity resin (Clontech) for 2 hours at 4ºC. Resins were washed extensively with lysis buffer containing 0.5% Triton X-100 and eluted in 50 mM NaAc (pH 5.0), 300 mM NaCl, 200 mM imidazole. Eluted proteins were dialyzed against PBS and diluted into equal amounts.

Electrophoretic mobility shift assay (EMSA)

Buffer C extracts (15 µg) from WI-38 cells and transfected hsf1-/- cells or recombinant HSF1 proteins were incubated with a ^{32}P-labeled oligonucleotide representing the proximal HSE from the human hsp70 promoter (9). The protein-DNA complexes were analyzed on a 4% native polyacrylamide gel

Oligonucleotide pull-down assay

The assay was performed essentially as previously described (8), with slight modifications. HeLa cells were lysed in cold lysis buffer [25 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM EDTA, 20 mM β-glycerophosphate, 20 mM p-nitro-phenyl-phosphate, 0.5% Triton X-100, 20 mM 100 μM sodium orthovanadate] containing protease inhibitors. Cell extracts were incubated with 1 μg of biotinylated oligonucleotide and proteins were allowed to bind the oligonucleotide for 30 minutes at room temperature. The oligonucleotides were precipitated with UltraLink streptavidin beads (Pierce) for 1h at 4°C. Bound fractions were washed three times with wash buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 0.1% Triton X-100), eluted with denaturing buffer and analyzed by western blotting.

The sequences for biotinylated oligonucleotides (Oligomer, Helsinki, Finland) were:
HSE forward: 5'-biotin-AACGAGAATCTTCGAGAATGGCT-3'
HSE reverse: 5'-AGCCATTCTCGAAGATTCTCGTT-3'

RT-PCR analysis

HeLa cells were harvested and RNA was generated using the Trizol reagent (Gibco-BRL, Gaithersburg, Maryland) according to the manufacturer's instructions. After the reverse transcription reaction, PCR was performed using the following PCR primers:

hsp90 forward: 5'-ACCGATTGGTGACATCTCCATGCT-3';
hsp90 reverse: 5'-CCAGGTGTTTCTTTGCTGCCATGT-3';
hsp70 forward: 5'-AGAGCCGAGCCGACAGAG-3';
hsp70 reverse: 5'-CACCTTGCCGTGTTGGAA-3';
hsp40 forward: 5'-CCCTCATGCCATGTTTGCTGAGTT-3';
hsp40 reverse: 5'-CCAAAGTTCACGTTGGTGAAGCCA-3';

hsp27 forward: 5’-AACGAGATCACCATCCCAGTCACCTT-3’;

hsp27 reverse: 5’-TAAGGCTTTACTTGGCGGCAGTCT-3’;

18S rRNA forward: 5’-CGTCTGCCCTATCAACTTTCG-3’;

18S rRNA reverse: 5’-TGCCTTCCTTGGATGTGGTAG-3’;

gapdh forward: 5’-CCACTCCTCCACCTTTGAC-3’;

gapdh reverse: 5’-ACCCTGTTGCTGTAGCCA-3’;

Quantitative real-time RT-PCR analysis

Analysis of hsp70 and hsp25 gene expression in transfected hsf1-/- cells was performed essentially as described (10). Briefly, RNA was isolated using the RNeasy kit (Qiagen). For each sample, 1 µg of RNA was treated with RQ1 DNase (Promega) and reverse-transcribed by using Moloney murine leukemia virus RNase H (–) (Promega). ABsolute QPCR ROX Mix (Advanced Biotechnologies) was used to prepare the reaction mixes. Relative quantities of hsp mRNAs were normalized against gapdh. All reactions were made in triplicates with samples derived from three biological repeats. The sequences for the primers and probes and their final concentrations were as follows:

hsp90 forward 5’-GGGAGCTCATCTCCAATTCA- 3’; (0.2 µM)

hsp90 reverse 5’-TGGGAATGAGATTGATGTGC -3’; (0.2 µM)

hsp90 probe 5’-6-FAM-GGAAGGAG-3´; (0.1 µM)

hsp70 forward, 5'-AGGTGC TGGACAAGTGCCAG-3'; (0.3 µM)

hsp70 reverse, 5'-AACTCCTCCTTGTCGGCCA-3'; (0.3 µM)

hsp70 probe, 5'-6-FAM-CATCTCCTGGCTGGACTCCAACACG-TAMRA-3'; (0.2 µM)

hsp40 forward 5’-ACCGCTATGGAGAGGAAGG-3’; (0.4 µM)

hsp40 reverse 5’-GAGGTACCATTAGCACCACCA-3’; (0.4 µM)

hsp40 probe 5’-6-FAM-GGAGGAAG-3’; (0.15 μM)

hsp25 forward, 5'-CACTGGCAAGCACGAAGAAAG-3'; (0.1 μM)

hsp25 reverse, 5'-GCGTGTATTTCCGGGTGAAG-3'; (0.1 μM)

hsp25 probe, 5'-6-FAM-ACCGAGAGATGTAGCCATGTTCGTCCTG-TAMRA-3'; (0.11 μM)

gapdh forward, 5'-TGCACCACCAACTGCTTAG-3'; (0.3 μM)

gapdh reverse, 5'-GGATGCAGGGATGATGTTC-3’; (0.3 μM)

gapdh probe, 5'-FAM-CAGAAGACTGTGGATGGCCCCTC-TAMRA 3'; (0.2 μM)

Confocal microscopy

HeLa cells growing on coverslips were kept at the control temperature or heat shocked for 1 hour at 42ºC. Cells were washed with PBS and simultaneously fixed and permeabilized in 3.7% paraformaldehyde containing 0.5% Triton X-100. Cells were washed twice with PBS and incubated in blocking solution (20% normal goat serum in PBS-0.05% Tween 20) for 1 hour. Primary antibodies [rabbit α-HSF1 (11), mouse α-Myc (Sigma)] were used at a 1:500 dilution in 5% BSA in PBS-0.05% Tween 20) overnight at 4ºC. After three washes with PBS-0.05% Tween 20, primary antibodies were detected using secondary goat α-mouse IgG (Rhodamine Red-X and Alexa Fluor 488, Molecular Probes) or goat α-rabbit IgG (Alexa Fluor 546, Molecular 488, Molecular Probes). All secondary antibodies were used at 1:500 dilutions in 5% BSA in PBS-0.05% Tween 20 for 1 hour at room temperature. After three washes, cells were mounted on slides in Vectashield containing DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories) and analyzed using a Zeiss LSM510 META confocal microscope. Images were further processed using Adobe Photoshop software.

Crosslinking

Cells were kept at 37ºC or heat shocked for 15 minutes at 42ºC and lysed in cold lysis buffer [25 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM β–glycerophosphate, 20 mM p-nitro-phenyl-phosphate, 100 µM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, protease inhibitor cocktail] and centrifuged at 4ºC for 10 minutes at 15,000 x g. 100 µg protein were crosslinked with 2 mM EGS [ethylene glycol bis(succinimidylsuccinate)] at room temperature for 15 minutes. After quenching of crosslinking by addition of 100 mM glycine, samples were boiled in denaturing buffer and analyzed on a 5% SDS-PAGE gel followed by western blotting.

Heat stress resistance assay

293T cells transfected with a vector control (Mock) or SIRT1 were compared for resistance to a 45°C heat shock. Cells were submerged in a 45°C water bath for 20 or 30 min and then allowed to recover at 37°C. Cells were assayed for percent cell death by trypan blue uptake 24 hours later.

Supplemental Figure Legends

Supplemental Figure 1. p300 and CBP acetylate HSF1 and p300 and SIRT1 bind to the hsp70 promoter *in vivo*.

(A) p300 and CBP, but not pCAF, induce HSF1 acetylation**.** 293T cells were transfected with Flag-HSF1 and p300, CBP, or pCAF, treated with HS and analyzed by acetylation assay. (B) p300 binds to the hsp70 promoter *in vivo*. Chromatin immunoprecipitation experiments performed at the indicated 42°C heat shock timepoints with and without the indicated recovery timepoints at 37°C show p300 binding to the hsp70 promoter. Chromatin was crosslinked, harvested, and immunoprecipitated with an antibody specific for p300 or HSF1. The samples were then analyzed by PCR with primers specific for the hsp70.1 promoter. Reactions were also performed using 1% of input and using no antibody. Upon heat shock, HSF1 and p300 are both recruited within one minute to the hsp70 promoter, and binding gradually attenuates during the recovery timepoints. (C) SIRT1 binds to the hsp70 promoter *in vivo*. Chromatin immunoprecipitation experiments performed at the indicated 42°C heat shock timepoints show SIRT1 binding to the hsp70 promoter. Chromatin was crosslinked, harvested, and immunoprecipitated with an antibody specific for HSF1 or SIRT1. The samples were then analyzed by PCR with primers specific for the hsp70 promoter. Control reactions were also performed using no antibody. SIRT1 binds to the hsp70 promoter under both basal and heat shock conditions.

Supplemental Figure 2. Bulk HSF1 phosphorylation in not a prerequisite for acetylation.

293T cells were cotransfected with Myc-HSF1 WT or a ten serine phosphomutant of HSF1 together with p300. Twenty-four hours after transfection, cells were treated with a one hour heat

shock at 42°C. Cell lysates were subjected to immunoprecipitation and acetylated HSF1 was detected using western blotting. Myc antibody was used to detect total HSF1.

Supplemental Figure 3. LC tandem mass spectrometry (MS/MS) shows that HSF1 is acetylated on at least nine lysines.

(A) HSF1 can be acetylated on nine lysines. Flag-HSF1, which was expressed and purified from 293T cells that had been cotransfected with p300 and subjected to either heat shock (42°C, 1 hour) or celastrol treatment (5 μM, 1 hour) and analyzed by LC-MS/MS. Positions of the identified acetylated lysine residues within murine HSF1 are shown. DBD, DNA-binding domain; L, linker region; HR-A/B and -C, heptad repeat domains; RD, regulatory domain; N, nuclear localization sequence; AD1 and AD2, activation domains. (B) Acetylated lysines are conserved. Sequence alignment of the acetylated lysines across multiple species was performed using T-COFFEE version 5.13. The acetylated lysines are shown in red, and amino acid numbers are indicated to the right. Hs, Homo sapiens (NP_005517); Mm, Mus musculus (NP_032322); Dr, Danio rerio (NP_571675); Xl, Xenopus laevis (NP_001084036.1); Dm, Drosophila melanogaster (P22813); Ce, Caenorhabditis elegans (NM_060630); Sc, Saccharomyces cerevisiae (P10961). (C-K) Mass spectrometry data for the nine acetylated sites within HSF1. MS/MS spectra were extracted and analyzed using the Mascot and Scaffold programs assuming digestion with trypsin. For each acetylated tryptic peptide identified, the mass spectrum generated from fragmentation of the HSF1-derived precursor ion is shown in the upper panel, and the corresponding fragment ion coverage is displayed in the lower panel. Acetylation is indicated by an additional mass of 42 Daltons. A low-mass ion at m/z 126 representing the acetylated lysine immonium ion minus NH3 was identified in most MS/MS spectra (labeled with asterisk) and used as further validation for acetylation (12).

Supplemental Figure 4. Human HSF1 K80 comes into close contact with the DNA phosphate backbone.

Using the coordinates supplied with the crystal structure of the *K. lactis* HSF DNA-binding domain complexed with an HSE (13), we threaded the aligned sequence of human HSF1 using Swiss Model. The side chain of K80 and the distance to the DNA phosphate backbone are shown in green. The boxed area in the upper panel is shown at a higher magnification in the lower panel.

Supplemental Figure 5. The HSF1 K80Q mutant is not defective in trimerization ability.

A prerequisite of stable HSF binding to DNA is the formation of trimeric HSF complexes. Therefore, it is conceivable that mutations of K80 disrupted proper HSF1 folding or the stress-induced formation of HSF trimers. To explore this possibility, we used a cross-linking assay to determine the formation of higher-order complexes upon heat shock. K562 cells were transfected with WT Myc-HSF1, Myc-HSF1 K80Q or Myc-HSF1 M391K, L395P, a previously characterized HSF1 mutant that is constitutively trimeric (14). Cells were treated with or without heat shock (HS, 42°C, 20 min) followed by treatment with the EGS crosslinking agent. Protein extracts were separated by SDS-PAGE and western blot analysis was performed with the indicated antibodies. The arrow indicates monomeric HSF1 and the arrowhead marks trimeric HSF1. Both WT and K80Q HSF1 underwent a monomer-to-trimer transition in response to heat shock, demonstrating that the mutations did not affect HSF1 trimerization.

Supplemental Figure 6. HSF1 DNA binding requires a lysine at residue 80.

EMSA reactions in hsf1-/- cells transfected with the indicated HSF1 construct and treated with or without heat shock (HS, 42°C 20 minutes) are shown. The oligonucleotide used as a probe

contains the proximal HSE from the human hsp70 promoter. Western blot analysis was performed on the same samples to show HSF1 and Hsc70 expression levels. Hsc70 is shown for equal loading. Mutation of K80 to A, H, N and T all interfered with the ability of HSF1 to bind to DNA upon heat shock.

Supplemental Figure 7. The formation of nuclear stress bodies requires a lysine at residue 80 of HSF1.

Confocal images of HeLa cells transfected with the indicated versions of Myc-HSF1 and treated with or without a one hour 42°C heat shock (HS) are shown. Exogenous HSF1 was stained using an α-Myc antibody (red) and DNA was stained using DAPI (blue). Bar = 5 μm. Mutation of HSF1 K80 to R, Q, A, H, N and T all impaired the ability of HSF1 to relocalize into characteristic nuclear stress bodies upon heat shock.

Supplemental Figure 8. HSF1 K80Q functions in a dominant-negative fashion.

Stable HSF1 binding to an HSE requires the simultaneous contact of three DNA-binding domains to three nGAAn repeats. As acetylation of one or two monomers could disrupt the binding of associated non-modified monomers, a small population of acetylated HSF1 DNA-binding domains is likely to have dominant negative effects. To investigate this issue, we transfected HeLa cells with WT or the K80Q mutant of Myc-HSF1 and double-stained the cells with antibodies against HSF1 or Myc to analyze the ability of endogenous HSF1 to form nSBs. Shown are confocal images of HeLa cells transfected with the indicated versions of Myc-HSF1 and treated with or without heat shock (HS, 42°C, 1 hour). HSF1 proteins were stained using antibodies against HSF1 (green) and Myc (red). DNA was stained using DAPI (blue). Bar = 5 μm. Whereas endogenous and exogenous WT HSF1 colocalized in nSBs upon heat shock, no nSBs could be detected with either antibody in cells expressing the K80Q mutant of HSF1.

Therefore, it is likely that the biological implications of K80 acetylation might be greater than the actual levels of K80-acetylated HSF1 molecules.

Supplemental Figure 9. Model of the HSF1 activation cycle.

Step 1: HSF1 in the resting state is an inert monomer that can be cytoplasmic or nuclear. Step 2: Upon stress, HSF1 forms DNA-binding homotrimers that bind to HSEs. Step 3; HSF1 acquires transcriptional activity. Step 4: HSF1 transcriptional activity is abrogated during attenuation. Attenuation has two regulated steps, including negative feedback from hsp expression that represses the transactivation function of DNA-bound HSF1 (not shown) and inhibition of DNA binding via acetylation of the DNA-binding domains of HSF1. SIRT1 regulates the attenuation phase of the HSR through prevention of HSF1 acetylation.

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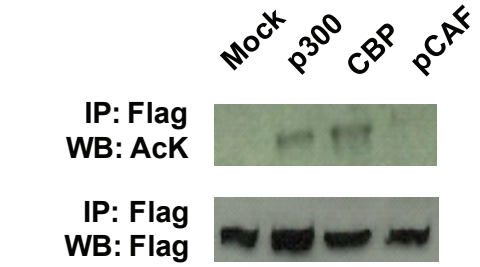
Supplemental Figure 1

A



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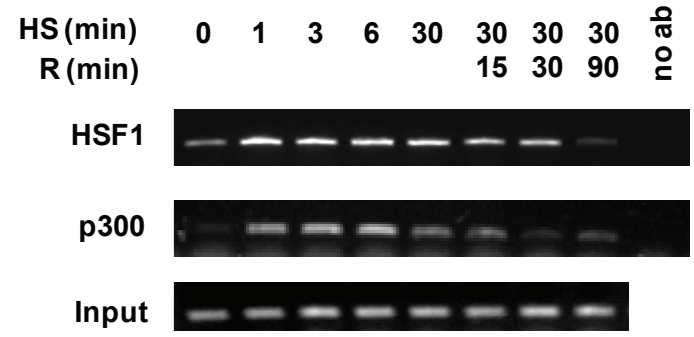
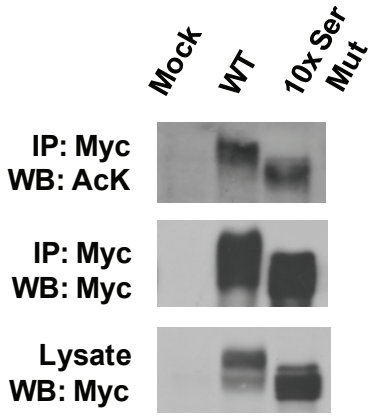


C

Supplemental Figure 2



Supplemental Figure 3

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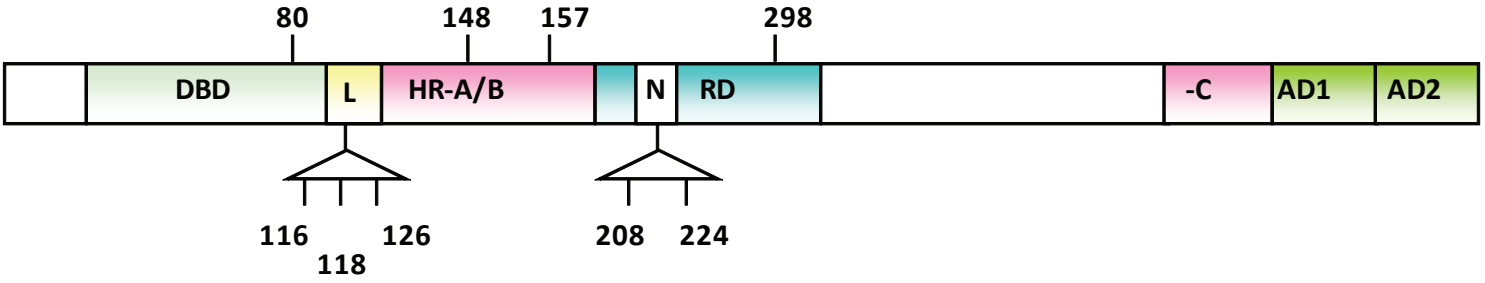

B

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Dr       HVFDQGRFSKEVLPKYFKHNNMASFVRQLNMYGFRKVVHIEQGGLVKPE--KDDTEFQHP  105
Xl       HVFDQGQFAKEVLPKYFKHNNMASFVRQLNMYGFRKVVHIEQGGLVKPE--RDDTEFQHP  99
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Sc       IVTNREEFVHQILPKYFKHSNFASFVRQLNMYGWHKVQDVKSGSIQSSS--DDKWQFENE  259

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Xl       YFIRGQEQLLENIKRKVNTMSATKSDEVKVRQDSVGKLISDVQSMKGKQESIDGRLLSMK  159
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Dm   ... ------------------SPCPSLEASP---SPVILIKTEPLTPSQ-SP---EQSP----  295
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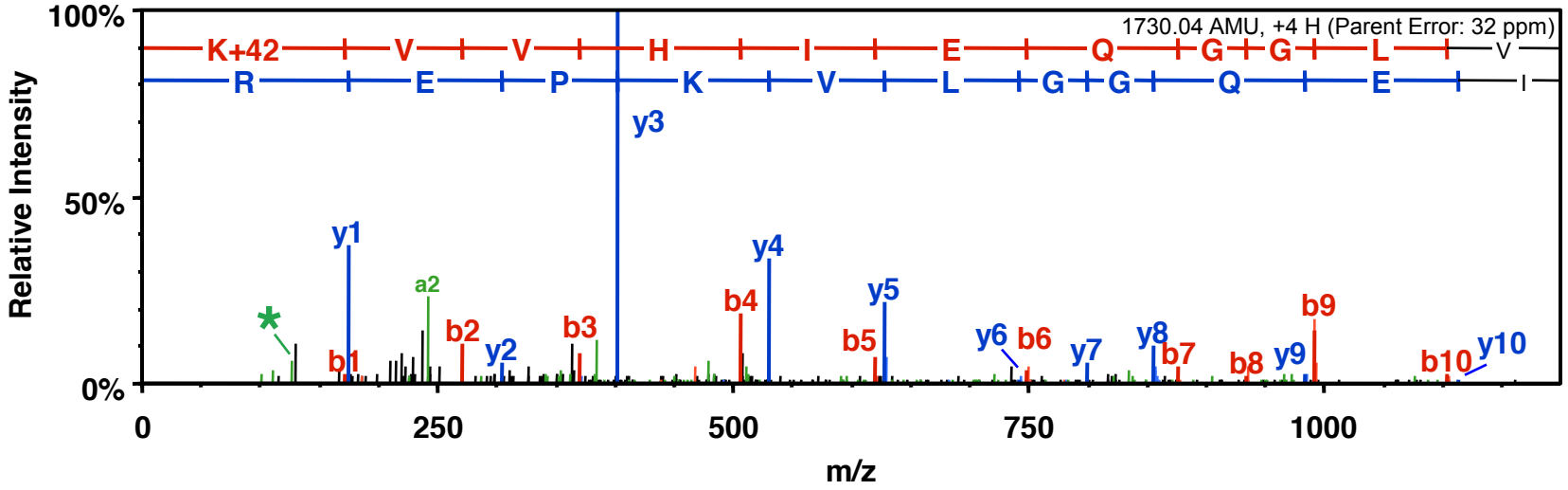
Supplemental Figure 3

K80

C



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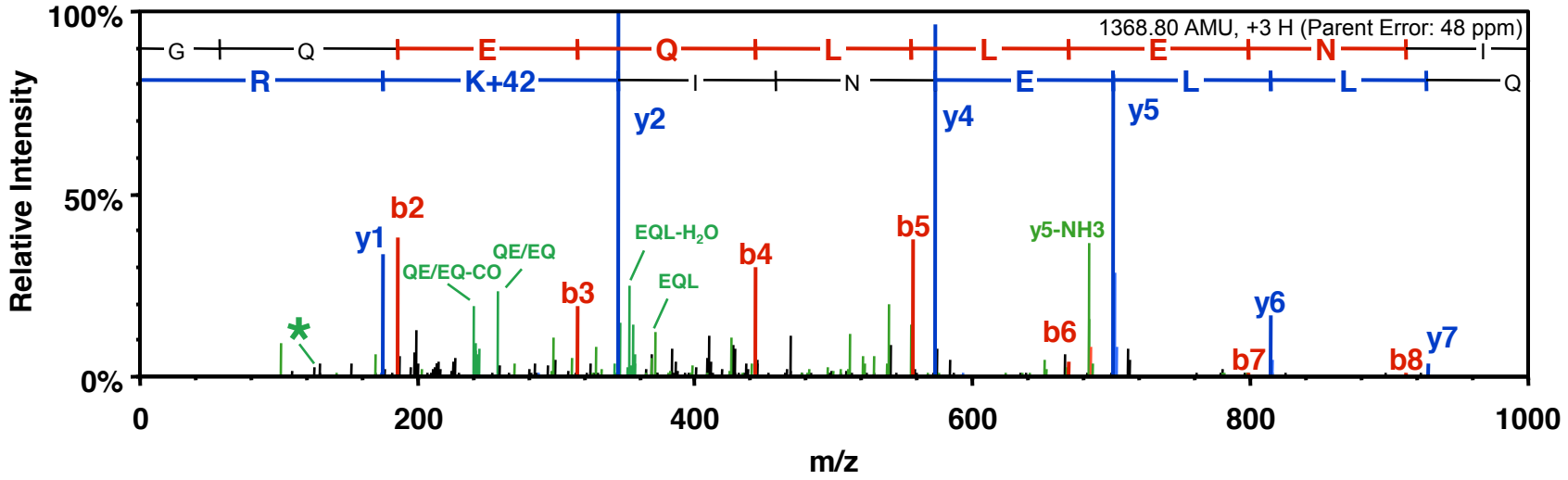
1	171.1	86.1	154.1		K+42	1731.0	866.0	1714.0	1713.0	15
2	270.2	135.6	253.2		V	1560.9	781.0	1543.9	1542.9	14
3	369.3	185.1	352.2		V	1461.8	731.4	1444.8	1443.8	13
4	506.3	253.7	489.3		H	1362.8	681.9	1345.7	1344.7	12
5	619.4	310.2	602.4		I	1225.7	613.4	1208.7	1207.7	11
6	748.4	374.7	731.4	730.4	E	1112.6	556.8	1095.6	1094.6	10
7	876.5	438.8	859.5	858.5	Q	983.6	492.3	966.5	965.6	9
8	933.5	467.3	916.5	915.5	G	855.5	428.3	838.5	837.5	8
9	990.5	495.8	973.5	972.5	G	798.5	399.8	781.5	780.5	7
10	1103.6	552.3	1086.6	1085.6	L	741.5	371.2	724.4	723.5	6
11	1202.7	601.9	1185.7	1184.7	V	628.4	314.7	611.4	610.4	5
12	1330.8	665.9	1313.8	1312.8	K	529.3	265.2	512.3	511.3	4
13	1427.8	714.4	1410.8	1409.8	P	401.2		384.2	383.2	3
14	1556.9	778.9	1539.9	1538.9	E	304.2		287.1	286.2	2
15	1731.0	866.0	1714.0	1713.0	R	175.1		158.1		1

Supplemental Figure 3

K116

D

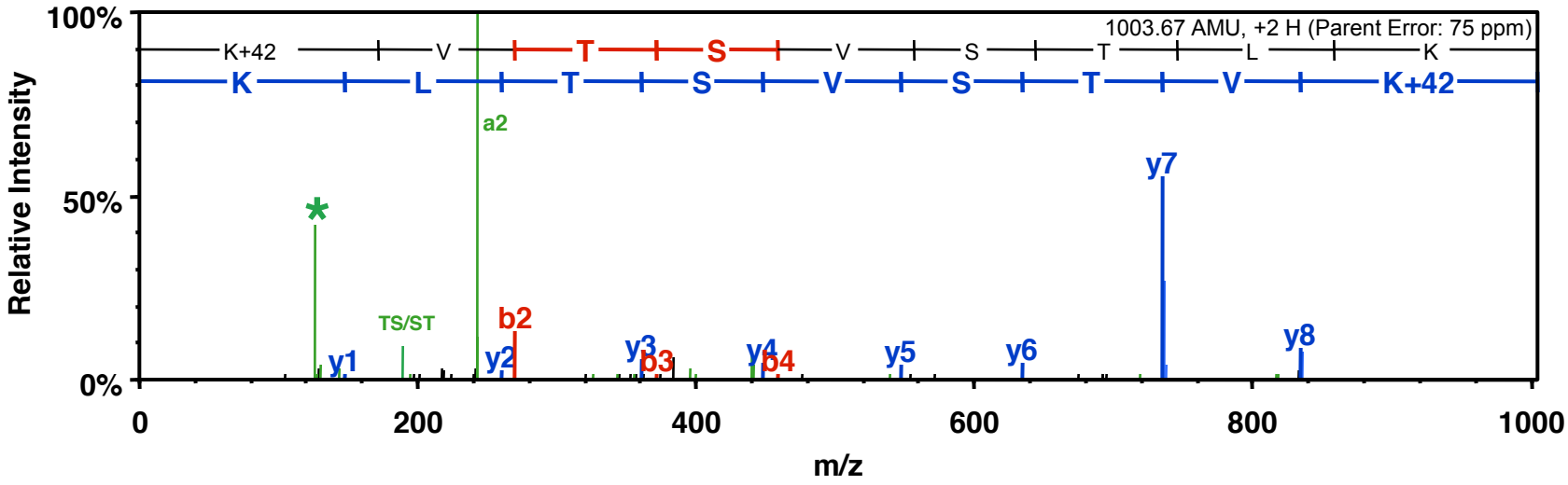


B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	58.0				G	1369.7	685.4	1352.7	1351.7	11
2	186.1		169.1		Q	1312.7	656.9	1295.7	1294.7	10
3	315.1		298.1	297.1	E	1184.7	592.8	1167.6	1166.7	9
4	443.2		426.2	425.2	Q	1055.6	528.3	1038.6	1037.6	8
5	556.3		539.3	538.3	L	927.6	464.3	910.5	909.6	7
6	669.4	335.2	652.3	651.4	L	814.5	407.7	797.5	796.5	6
7	798.4	399.7	781.4	780.4	E	701.4	351.2	684.4	683.4	5
8	912.4	456.7	895.4	894.4	N	572.4	286.7	555.3		4
9	1025.5	513.3	1008.5	1007.5	I	458.3	229.7	441.3		3
10	1195.6	598.3	1178.6	1177.6	K+42	345.2	173.1	328.2		2
11	1369.7	685.4	1352.7	1351.7	R	175.1		158.1		1

Supplemental Figure 3

K118

E

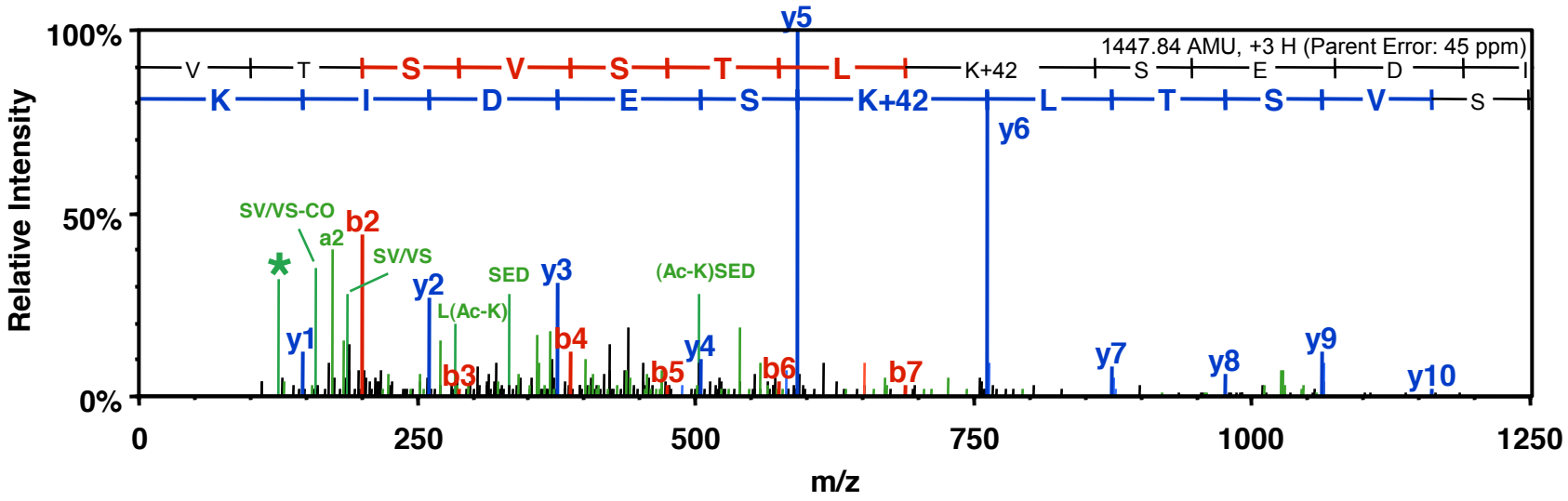


B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	171.1	86.1	154.1		K+42	1004.6	502.8	987.6	986.6	9
2	270.2	135.6	253.2		V	834.5	417.8	817.5	816.5	8
3	371.2	186.1	354.2	353.2	T	735.4	368.2	718.4	717.4	7
4	458.3	229.6	441.2	440.3	S	634.4	317.7	617.4	616.4	6
5	557.3	279.2	540.3	539.3	V	547.4		530.3	529.3	5
6	644.4	322.7	627.3	626.4	S	448.3		431.3	430.3	4
7	745.4	373.2	728.4	727.4	T	361.3		344.2	343.2	3
8	858.5	429.8	841.5	840.5	L	260.2		243.2		2
9	1004.6	502.8	987.6	986.6	K	147.1		130.1		1

Supplemental Figure 3

K126

F

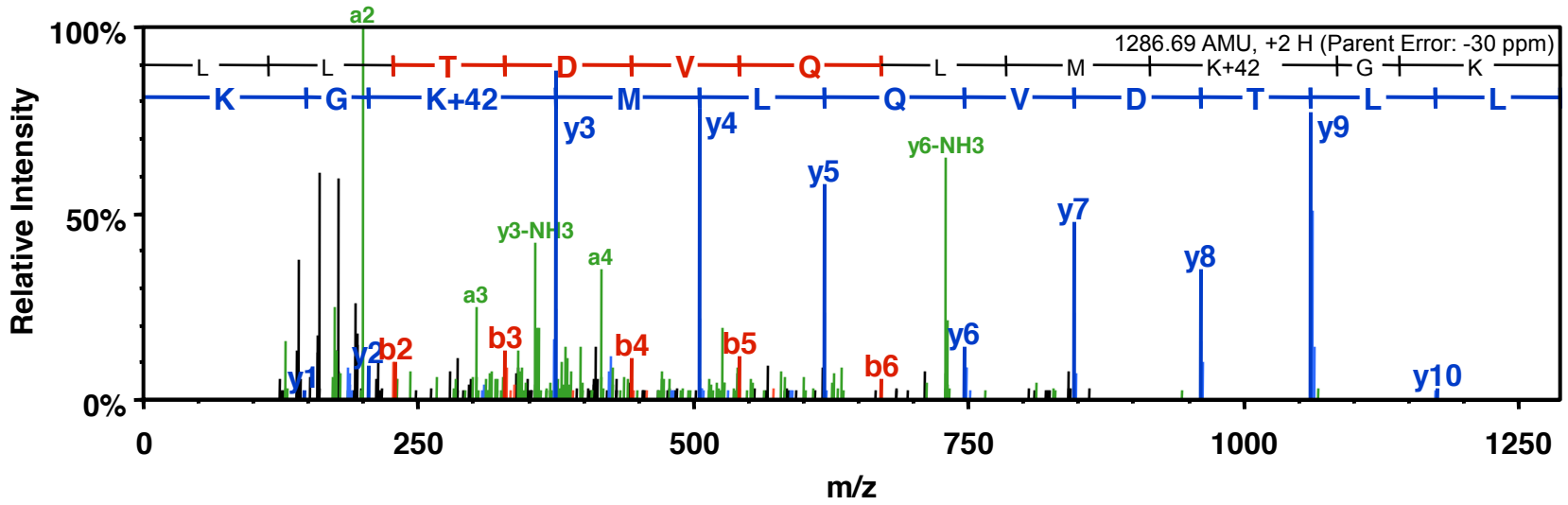


B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	100.1				V	1448.8	724.9	1431.8	1430.8	13
2	201.1			183.1	T	1349.7	675.4	1332.7	1331.7	12
3	288.2			270.1	S	1248.7	624.8	1231.6	1230.7	11
4	387.2			369.2	V	1161.6	581.3	1144.6	1143.6	10
5	474.3			456.3	S	1062.6	531.8	1045.5	1044.6	9
6	575.3	288.2		557.3	T	975.5	488.3	958.5	957.5	8
7	688.4	344.7		670.4	L	874.5	437.8	857.5	856.5	7
8	858.5	429.8	841.5	840.5	K+42	761.4	381.2	744.4	743.4	6
9	945.5	473.3	928.5	927.5	S	591.3		574.3	573.3	5
10	1074.6	537.8	1057.5	1056.6	E	504.3		487.2	486.3	4
11	1189.6	595.3	1172.6	1171.6	D	375.2		358.2	357.2	3
12	1302.7	651.8	1285.7	1284.7	I	260.2		243.2		2
13	1448.8	724.9	1431.8	1430.8	K	147.1		130.1		1

Supplemental Figure 3

K148

G

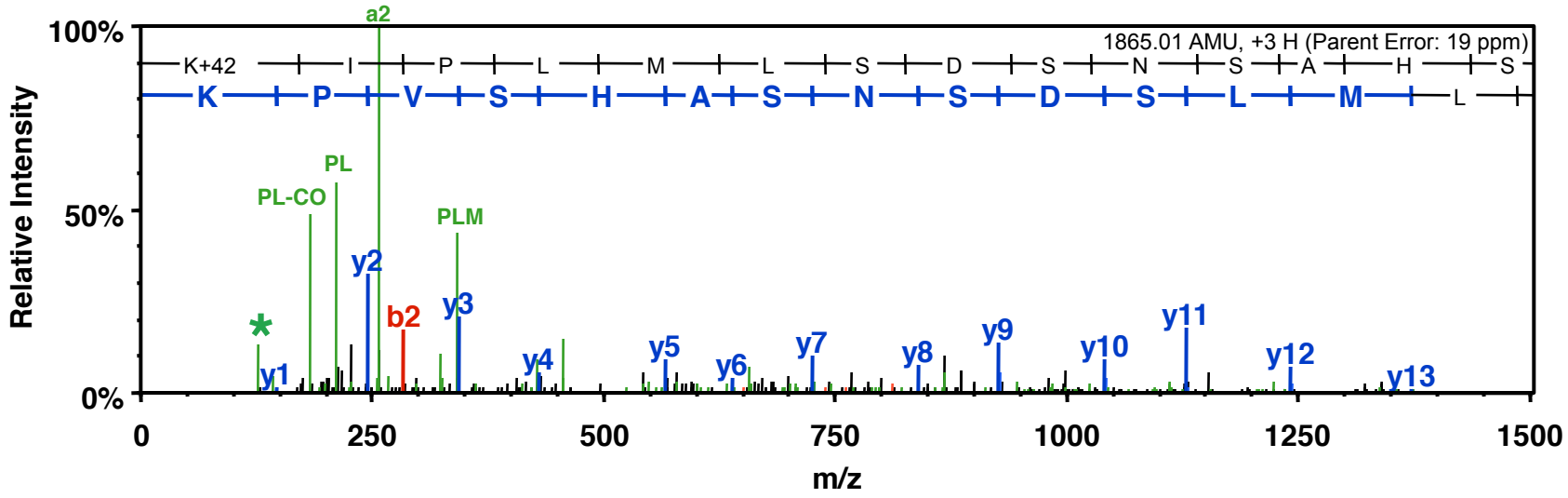


B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	114.1				L	1287.7	644.4	1270.7	1269.7	11
2	227.2				L	1174.7	587.8	1157.6	1156.6	10
3	328.2			310.2	T	1061.6	531.3	1044.5	1043.6	9
4	443.3			425.2	D	960.5	480.8	943.5	942.5	8
5	542.3			524.3	V	845.5	423.3	828.5		7
6	670.4	335.7	653.4	652.4	Q	746.4	373.7	729.4		6
7	783.5	392.2	766.4	765.5	L	618.4	309.7	601.3		5
8	914.5	457.8	897.5	896.5	M	505.3	253.1	488.3		4
9	1084.6	542.8	1067.6	1066.6	K+42	374.2	187.6	357.2		3
10	1141.6	571.3	1124.6	1123.6	G	204.1		187.1		2
11	1287.7	644.4	1270.7	1269.7	K	147.1		130.1		1

Supplemental Figure 3

K208

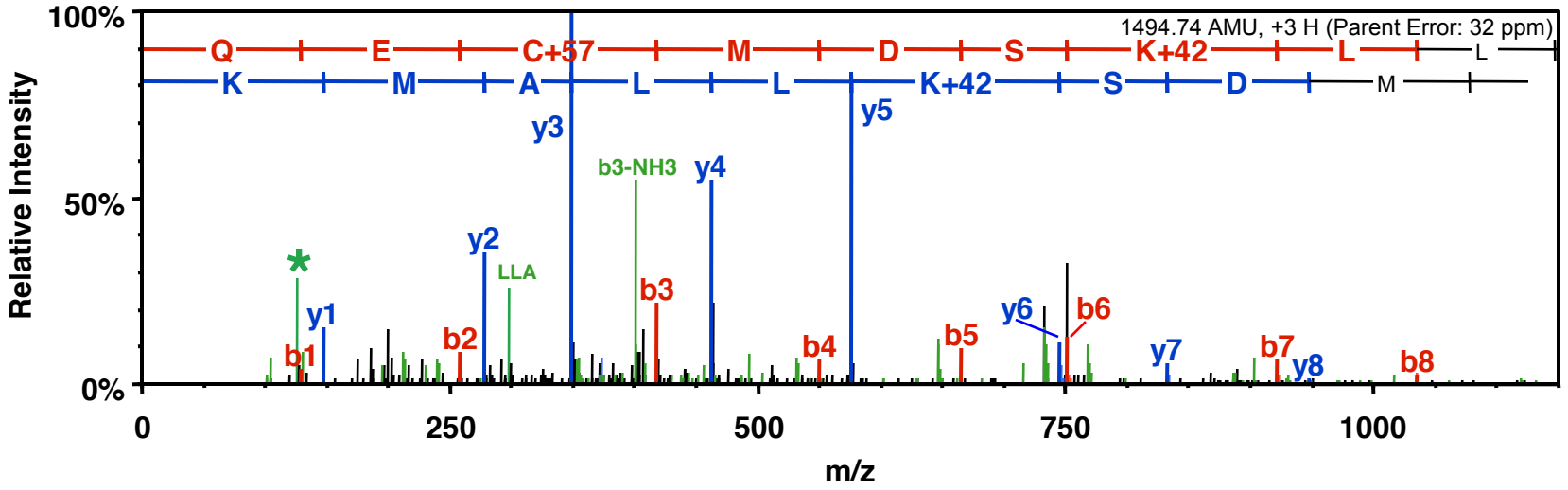
H



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	171.1	86.1	154.1		K+42	1866.0	933.5	1849.0	1848.0	17
2	284.2	142.6	267.2		I	1695.9	848.4	1678.9	1677.9	16
3	381.3	191.1	364.2		P	1582.8	791.9	1565.8	1564.8	15
4	494.3	247.7	477.3		L	1485.7	743.4	1468.7	1467.7	14
5	625.4	313.2	608.4		M	1372.7	686.8	1355.6	1354.6	13
6	738.5	369.7	721.4		L	1241.6	621.3	1224.6	1223.6	12
7	825.5	413.3	808.5	807.5	S	1128.5	564.8	1111.5	1110.5	11
8	940.5	470.8	923.5	922.5	D	1041.5	521.3	1024.5	1023.5	10
9	1027.5	514.3	1010.5	1009.5	S	926.5	463.7	909.4	908.5	9
10	1141.6	571.3	1124.6	1123.6	N	839.4	420.2	822.4	821.4	8
11	1228.6	614.8	1211.6	1210.6	S	725.4	363.2	708.4	707.4	7
12	1299.7	650.3	1282.6	1281.7	A	638.4	319.7	621.3	620.4	6
13	1436.7	718.9	1419.7	1418.7	H	567.3	284.2	550.3	549.3	5
14	1523.8	762.4	1506.7	1505.7	S	430.3		413.2	412.3	4
15	1622.8	811.9	1605.8	1604.8	V	343.2		326.2		3
16	1719.9	860.4	1702.9	1701.9	P	244.2		227.1		2
17	1866.0	933.5	1849.0	1848.0	K	147.1		130.1		1

Supplemental Figure 3

I

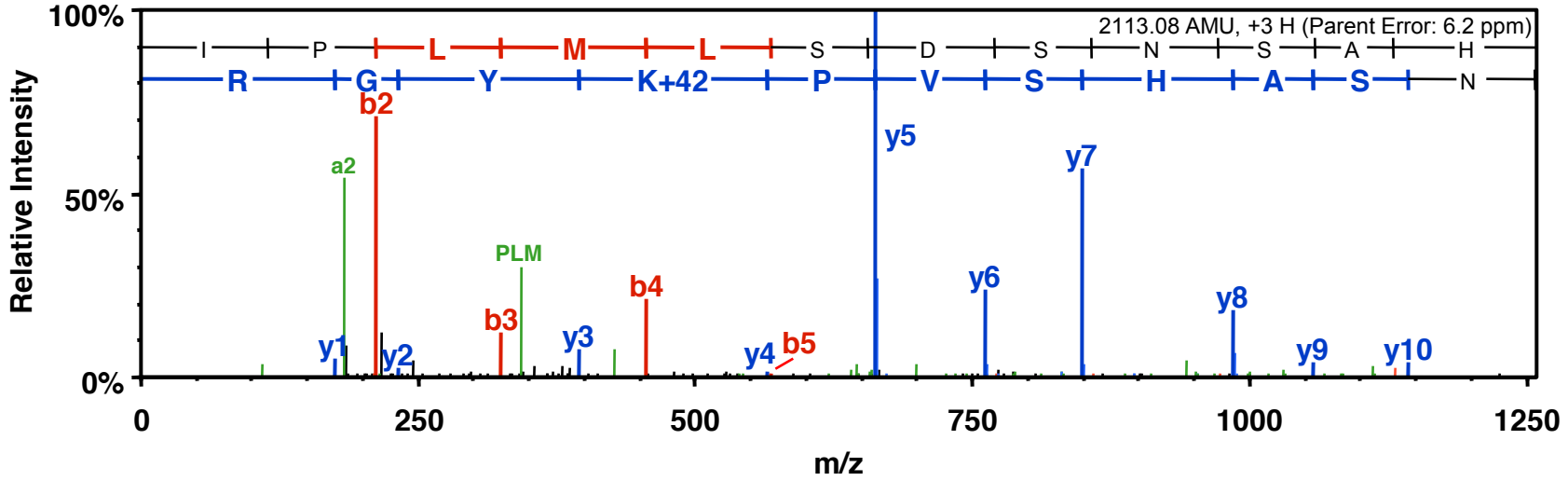


B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	129.1		112.0		Q	1495.7	748.4	1478.7	1477.7	12
2	258.1		241.1	240.1	E	1367.6	684.3	1350.6	1349.6	11
3	418.1		401.1	400.1	C+57	1238.6	619.8	1221.6	1220.6	10
4	549.2		532.1	531.2	M	1078.6	539.8	1061.5	1060.5	9
5	664.2		647.2	646.2	D	947.5	474.3	930.5	929.5	8
6	751.2	376.1	734.2	733.2	S	832.5	416.8	815.5	814.5	7
7	921.3	461.2	904.3	903.3	K+42	745.5	373.2	728.4		6
8	1034.4	517.7	1017.4	1016.4	L	575.4		558.3		5
9	1147.5	574.3	1130.5	1129.5	L	462.3		445.3		4
10	1218.5	609.8	1201.5	1200.5	A	349.2		332.2		3
11	1349.6	675.3	1332.6	1331.6	M	278.2		261.1		2
12	1495.7	748.4	1478.7	1477.7	K	147.1		130.1		1

Supplemental Figure 3

K224

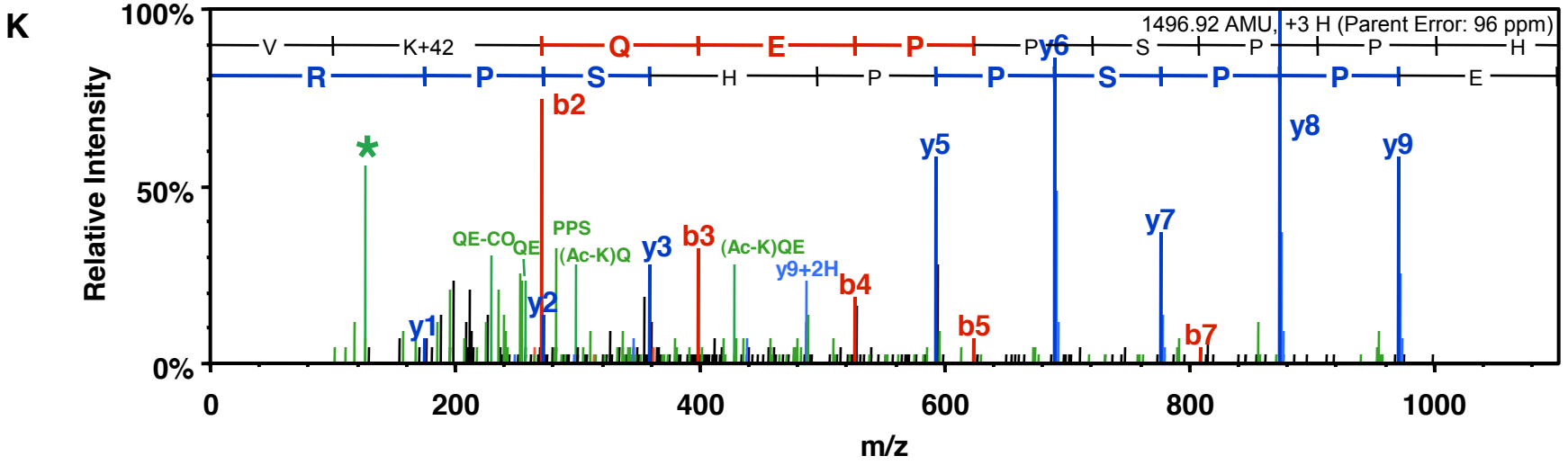
J



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	114.1				I	2114.1	1057.5	2097.0	2096.1	19
2	211.1				P	2001.0	1001.0	1984.0	1983.0	18
3	324.2				L	1903.9	952.5	1886.9	1885.9	17
4	455.3				M	1790.9	895.9	1773.8	1772.8	16
5	568.4				L	1659.8	830.4	1642.8	1641.8	15
6	655.4	328.2		637.4	S	1546.7	773.9	1529.7	1528.7	14
7	770.4	385.7		752.4	D	1459.7	730.4	1442.7	1441.7	13
8	857.4	429.2		839.4	S	1344.7	672.8	1327.6	1326.7	12
9	971.5	486.3	954.5	953.5	N	1257.6	629.3	1240.6	1239.6	11
10	1058.5	529.8	1041.5	1040.5	S	1143.6	572.3	1126.6	1125.6	10
11	1129.6	565.3	1112.5	1111.5	A	1056.6	528.8	1039.5	1038.5	9
12	1266.6	633.8	1249.6	1248.6	H	985.5	493.3	968.5	967.5	8
13	1353.7	677.3	1336.6	1335.6	S	848.5	424.7	831.4	830.5	7
14	1452.7	726.9	1435.7	1434.7	V	761.4	381.2	744.4		6
15	1549.8	775.4	1532.7	1531.8	P	662.4	331.7	645.3		5
16	1719.9	860.4	1702.9	1701.9	K+42	565.3	283.2	548.3		4
17	1882.9	942.0	1865.9	1864.9	Y	395.2		378.2		3
18	1940.0	970.5	1922.9	1922.0	G	232.1		215.1		2
19	2114.1	1057.5	2097.0	2096.1	R	175.1		158.1		1

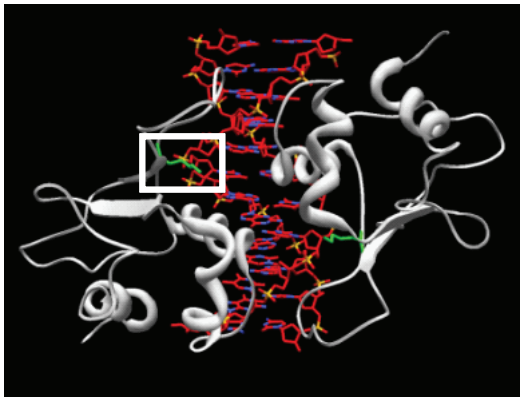
Supplemental Figure 3

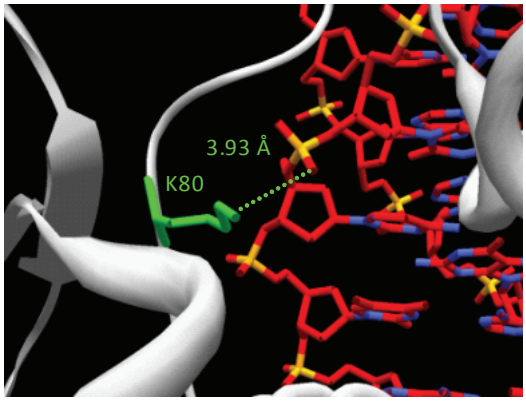
K298



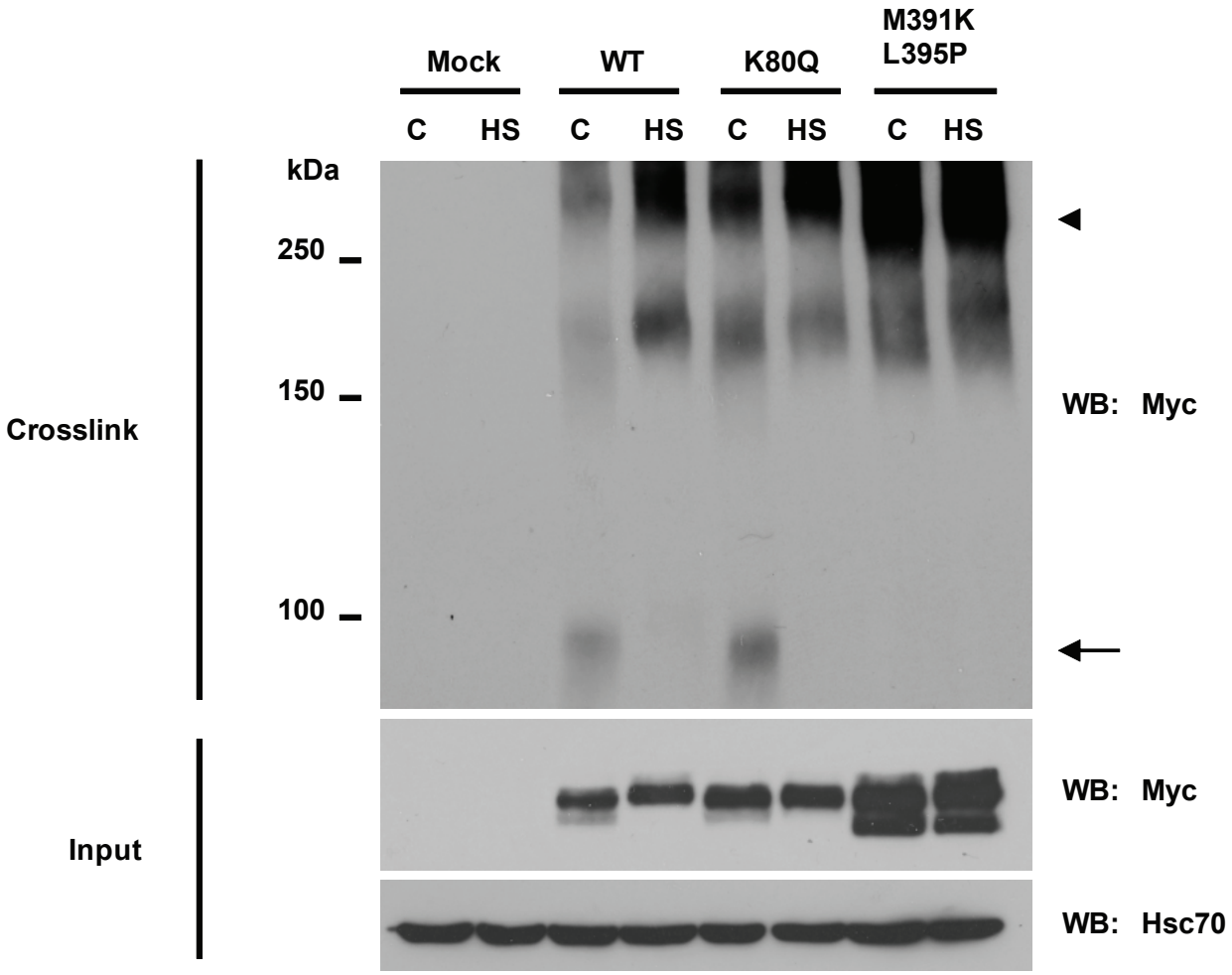
B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	100.1				V	1497.8	749.4	1480.8	1479.8	13
2	270.2	135.6	253.2		K+42	1398.7	699.9	1381.7	1380.7	12
3	398.2	199.6	381.2		Q	1228.6	614.8	1211.6	1210.6	11
4	527.3	264.1	510.3	509.3	E	1100.5	550.8	1083.5	1082.5	10
5	624.3	312.7	607.3	606.3	P	971.5	486.3	954.5	953.5	9
6	721.4	361.2	704.4	703.4	P	874.5	437.7	857.4	856.4	8
7	808.4	404.7	791.4	790.4	S	777.4	389.2	760.4	759.4	7
8	905.5	453.2	888.5	887.5	P	690.4	345.7	673.3	672.4	6
9	1002.5	501.8	985.5	984.5	P	593.3	297.2	576.3	575.3	5
10	1139.6	570.3	1122.6	1121.6	H	496.3	248.6	479.2	478.3	4
11	1226.6	613.8	1209.6	1208.6	S	359.2		342.2	341.2	3
12	1323.7	662.3	1306.6	1305.7	P	272.2		255.2		2
13	1497.8	749.4	1480.8	1479.8	R	175.1		158.1		1

Supplemental Figure 4

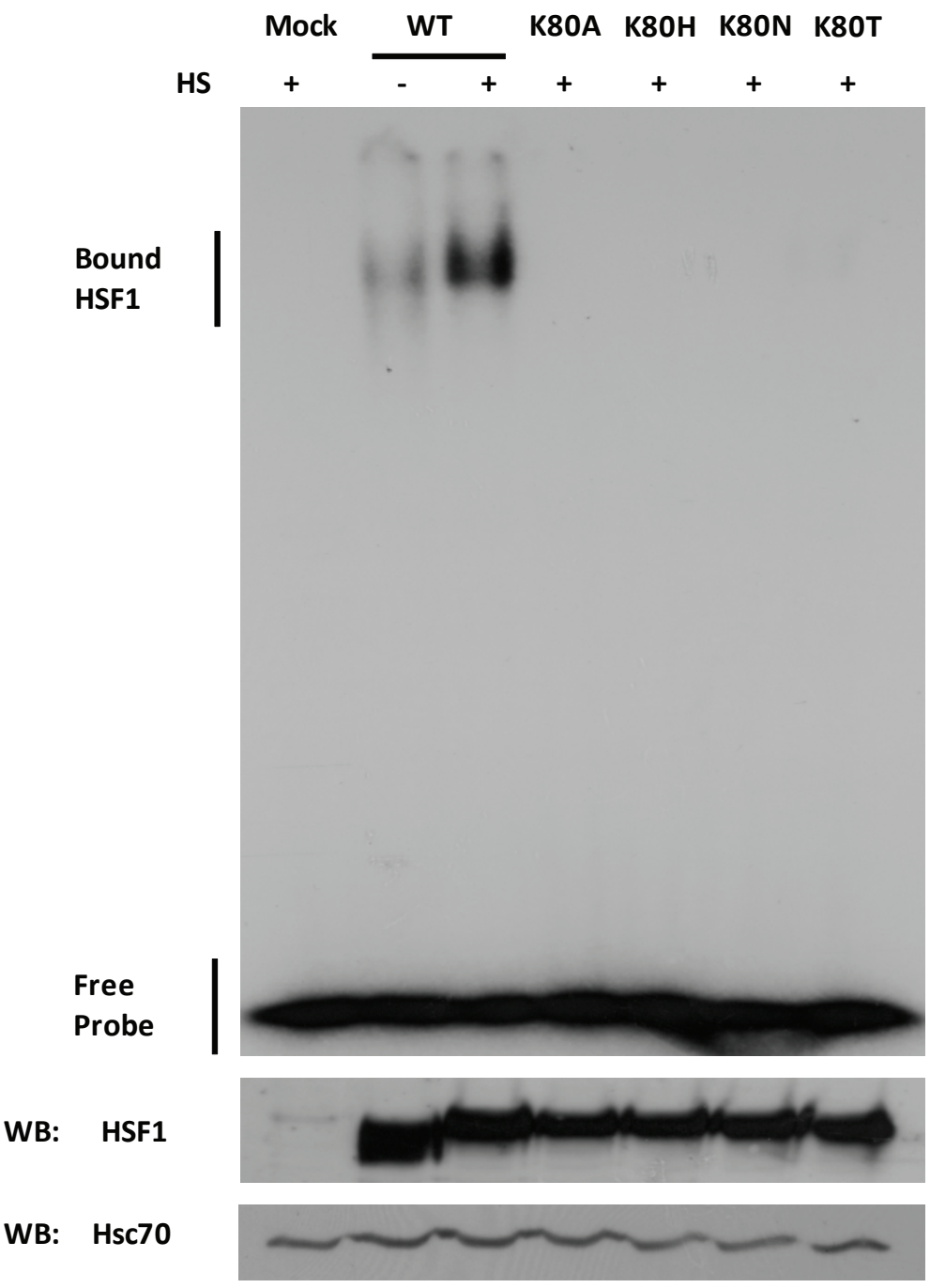




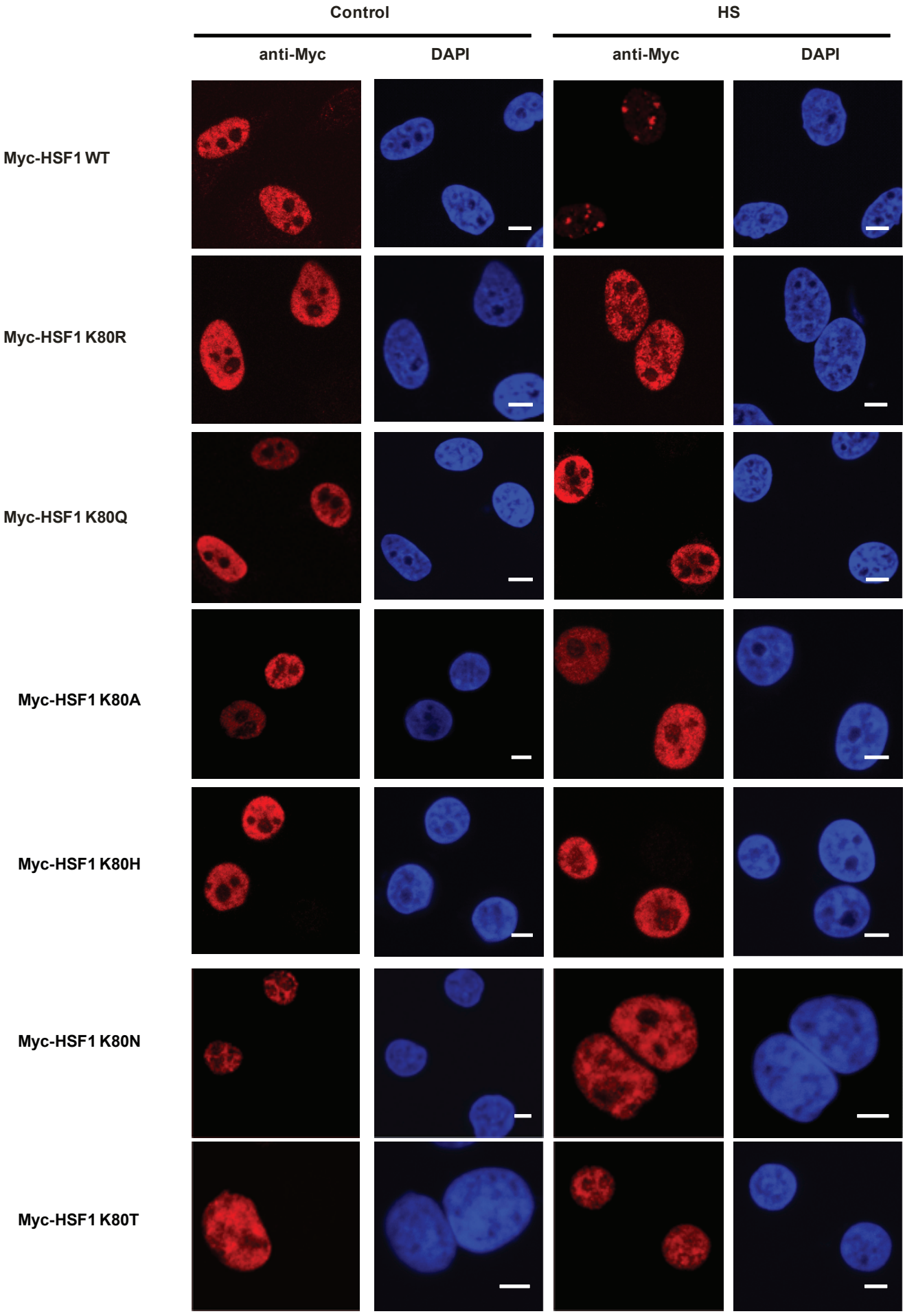
Supplemental Figure 5



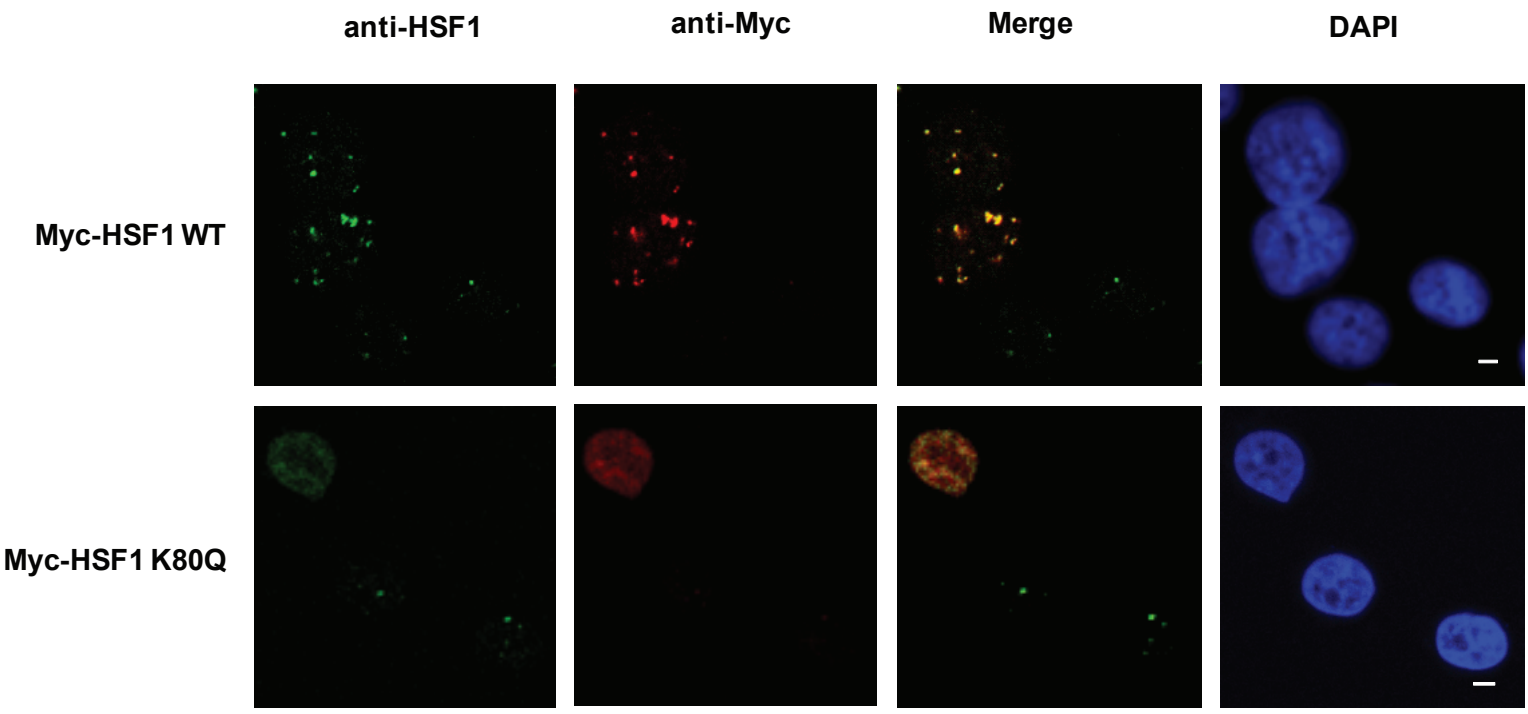
Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9