SUPPLEMENTARY TABLE

TABLE S1. **Crosslinks involving adjacent peptides on GST**. Eleven crosslinks were reported collectively by StavroX and Crux involving adjacent tryptic peptides. Since GST forms homodimers, both intra-subunit and inter-subunit interpretations were considered for each crosslink. X's indicate that the crosslinked sites occur within 3-6 residues and so the associated Cα-Cα distance will be within 12 Å for most conformations. These interpretations therefore will usually not contribute useful distance constraints, even if they are true. Overall, two putative crosslinked peptides are clearly false positives and most others are uninformative or false positives.

Position A	Peptide A	Position B	PeptideB	Cα-Cα Distance	
				Intra	Inter
19-35	LLLEYLEEKYEEHLY[E]R	36-42	DEGD[K]WR	x	49.8
28-35	YEEHLY[E]R	36-42	DEGD[K]WR	x	49.8
36-40	DEG[D]K	41-45	WRN[K]K	x	52
36-42	DEG[D]KWR	43-45	N[K]K	x	52
43-45	N[K]K	46-64	F[E]LGLEFPNLPYYIDGDVK	x	52.3
182-191	RIEAIPQI[D]K	192-197	YL[K]SSK	x	58.1
183-191	IEAIPQI[D]K	192-197	YL[K]SSK	x	58.1
10-18	I[K]GLVQPTR	19-27	LLL[E]YLEEK	13.6	40.3
109-119	IAYS[K]DFETLK	120-125	V[D]FLSK	14.5	15.6
19-27	LLL[E]YLEEK	28-42	YEEHLYERDEGD[K]WR	26.7	45.5
19-35	LLLEYLEE[K]YEEHLYER	36-40	[D]EGDK	27.5	54.5

TABLE S2. **The 29 most abundant proteins found in the intact membrane sample**. Proteins were required to have at least 5 peptides and 10 spectral counts in the analysis of the untreated control sample. Proteins were than ranked by relative abundance based on spectral count normalized to molecular weight (M.W.).

Uniprot ID	Protein Description	Gene ID	Peptide Count	Spectral Count	Sequence Coverage (%)	Sequence Length	M.W. (kDa)
P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	13	123	55.2	335	36
P02730	Band 3 anion transport protein	SLC4A1	25	323	45.7	912	102
P02549	Spectrin alpha chain, erythrocyte	SPTA1	115	593	60.5	2419	280
P11277	Spectrin beta chain, erythrocyte	SPTB	108	537	55	2137	268
P16157	Ankyrin-1	ANK1	68	365	58.9	1881	217
P69905	Hemoglobin subunit alpha	HBA1;HBA2	6	21	62.7	142	15
P11171	Protein 4.1	EPB41	23	131	38.9	864	97
P68871	Hemoglobin subunit beta	HBB	9	20	67.3	147	16
P60712;P63261	Actin, cytoplasmic 1;Actin, cytoplasmic 2	ACTB;ACTG1	18	50	56	375	42
P16452	Erythrocyte membrane protein band 4.2	EPB42	20	79	43	691	77
P27105	Erythrocyte band 7 integral membrane protein	STOM	10	27	38.5	288	32
Q08495	Dematin	EPB49	12	25	41.3	405	46
P11166	Solute carrier family 2, facilitated glucose transporter member 1	SLC2A1	10	24	23.8	492	54
Q00013	55 kDa erythrocyte membrane protein	MPP1	12	22	41	466	52
P35612	Beta-adducin	ADD2	21	33	44.1	726	81
O75955	Flotillin-1	FLOT1	10	18	36.3	427	47
P28289	Tropomodulin-1	TMOD1	11	14	38.2	359	41
P35611	Alpha-adducin	ADD1	13	28	33.6	737	81
Q9UKV8	Protein argonaute-2	EIF2C2	18	26	32	859	97
E7EMK3	Flotillin-2	FLOT2	8	14	23.8	483	53
Q96PL5	Erythroid membrane-associated protein	ERMAP	8	12	29.7	475	53
P11142	Heat shock cognate 71 kDa protein	HSPA8	13	14	38.9	646	71
Q00610	Clathrin heavy chain 1	CLTC	31	37	34.7	1675	192
B5U6Z5	Kell blood group glycoprotein	KEL	12	16	26.4	732	83
P55072	Transitional endoplasmic reticulum ATPase	VCP	14	17	33.3	806	89
P08107	Heat shock 70 kDa protein 1A/1B	HSPA1A; HSPA1B	9	10	28.2	641	70
Q13200	26S proteasome non-ATPase regulatory subunit 2	PSMD2	10	12	19.9	908	100
K7BW74	26S proteasome non-ATPase regulatory subunit 1	PSMD1	10	10	21	959	107

TABLE S3. **The 19 most abundant proteins found in the membrane cytoskeleton**. Proteins were required to have at least 5 peptides and 5 spectral counts in the analysis of the untreated control sample. Proteins were than ranked by relative abundance based on spectral count normalized to molecular weight (M.W.).

Uniprot ID	Protein Description	Gene ID	Peptide Count	Spectral Count	Sequence Coverage (%)	Sequence Length	M.W. (kDa)
P02549	Spectrin alpha chain, erythrocyte	SPTA1	118	525	60.5	2419	280
P11277	Spectrin beta chain, erythrocyte	SPTB	110	497	55	2137	268
P60712;P63261	Actin, cytoplasmic 1;Actin, cytoplasmic 2	ACTB;ACTG1	20	45	56	375	42
P02730	Band 3 anion transport protein	SLC4A1	26	107	45.7	912	102
P16157	Ankyrin-1	ANK1	67	182	58.9	1881	217
P11171	Protein 4.1	EPB41	13	46	38.9	864	97
O75955	Flotillin-1	FLOT1	8	15	36.3	427	47
P27105	Erythrocyte band 7 integral membrane protein	STOM	8	9	38.5	288	32
P16452	Erythrocyte membrane protein band 4.2	EPB42	15	21	43	691	77
E7EMK3	Flotillin-2	FLOT2	8	10	23.8	483	53
P28289	Tropomodulin-1	TMOD1	5	7	38.2	359	41
P11142	Heat shock cognate 71 kDa protein	HSPA8	11	11	38.9	646	71
P35611	Alpha-adducin	ADD1	8	12	33.6	737	81
Q96PL5	Erythroid membrane-associated protein	ERMAP	5	6	29.7	475	53
B5U6Z5	Kell blood group glycoprotein	KEL	6	8	26.4	732	83
P35612	Beta-adducin	ADD2	5	7	44.1	726	81
Q9UKV8	Protein argonaute-2	EIF2C2	5	5	32	859	97
P35579	Myosin-9	MYH9	9	9	12.9	1960	227

SUPPLEMENTARY FIGURES

FIGURE S1. **Label-free comparison**. (A) Label-free comparison between crosslinked and control samples greatly narrow down the list of candidate precursor masses. Only signals enriched in crosslinked samples are retained for downstream analysis. (B) Weak unrelated MS signals from the control (red peak profile, indicated by the arrows) will sometimes overlap crosslinked peptide precursors by random chance in complex mixtures. Therefore a 10-fold enrichment threshold rather than only crosslink-specific signals needs to be considered.

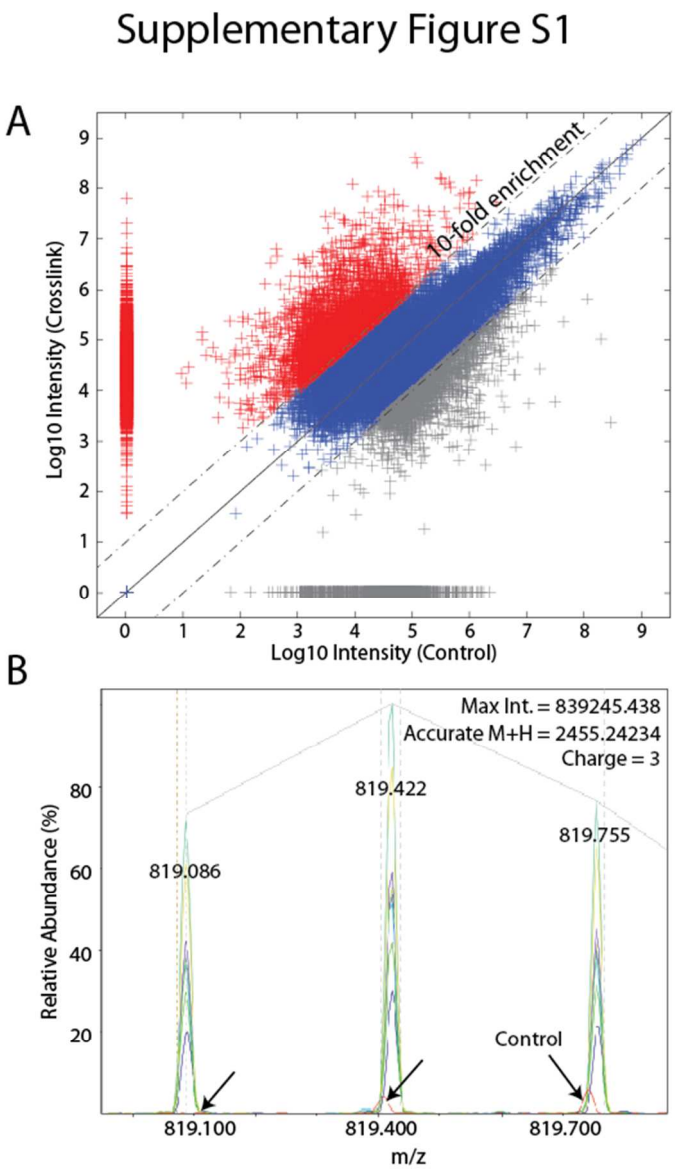


FIGURE S2. **Effect of removing MS/MS peaks with unassigned charge states**. Using high-resolution MS/MS information, we can de-isotope the majority of the peaks to determine their charge states and monoisotopic masses. The remaining peaks can either be removed from further analysis since they are ambiguous and could result in erroneous matches or be retained and allowed to assume all possible charge state assignments. The two alternative strategies were compared using the GST dataset and only minor differences in crosslink identification performance were observed. Scatter plots show the scores of positive and negative crosslinked peptides (A) using all MS/MS peaks or (B) using only peaks with assigned charge states. The corresponding areas under the ROC curve are 0.9931 and 0.9934, respectively.

Supplementary Figure S2

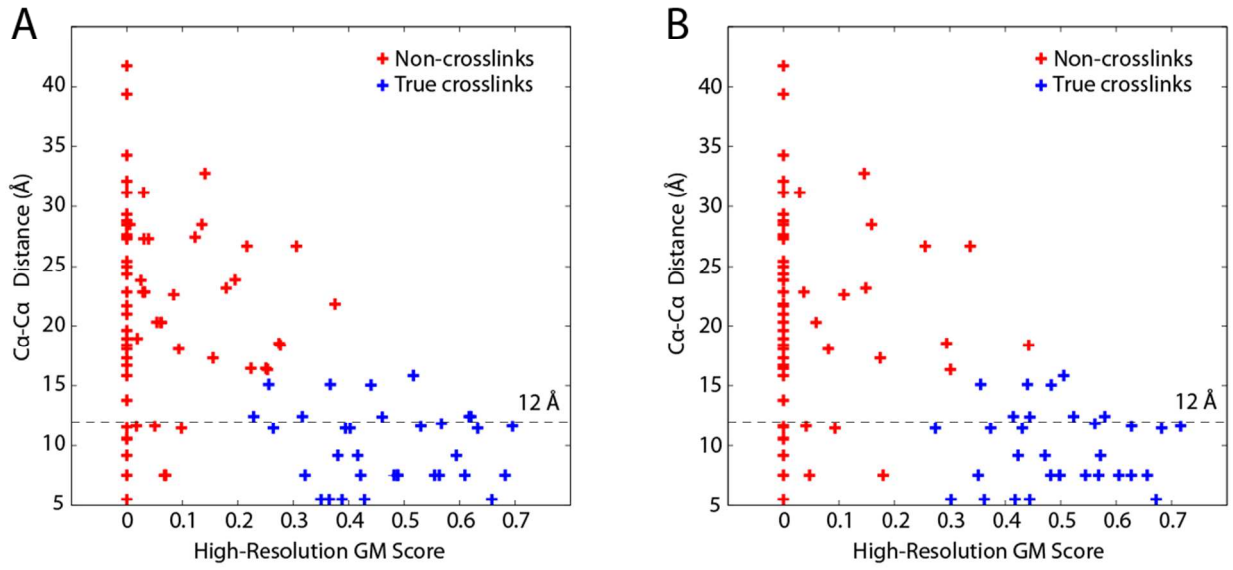


FIGURE S3. **Consistency of crosslink identification across software packages**. The histogram shows the number of true crosslinked peptides that were reported by one, two, three, four, or all software packages. At the unique precursor level, crosslinked peptides with distinct charge states or methionine oxidation states were counted as separate entries. At the unique sequence level, only differences in amino acid sequences were counted.

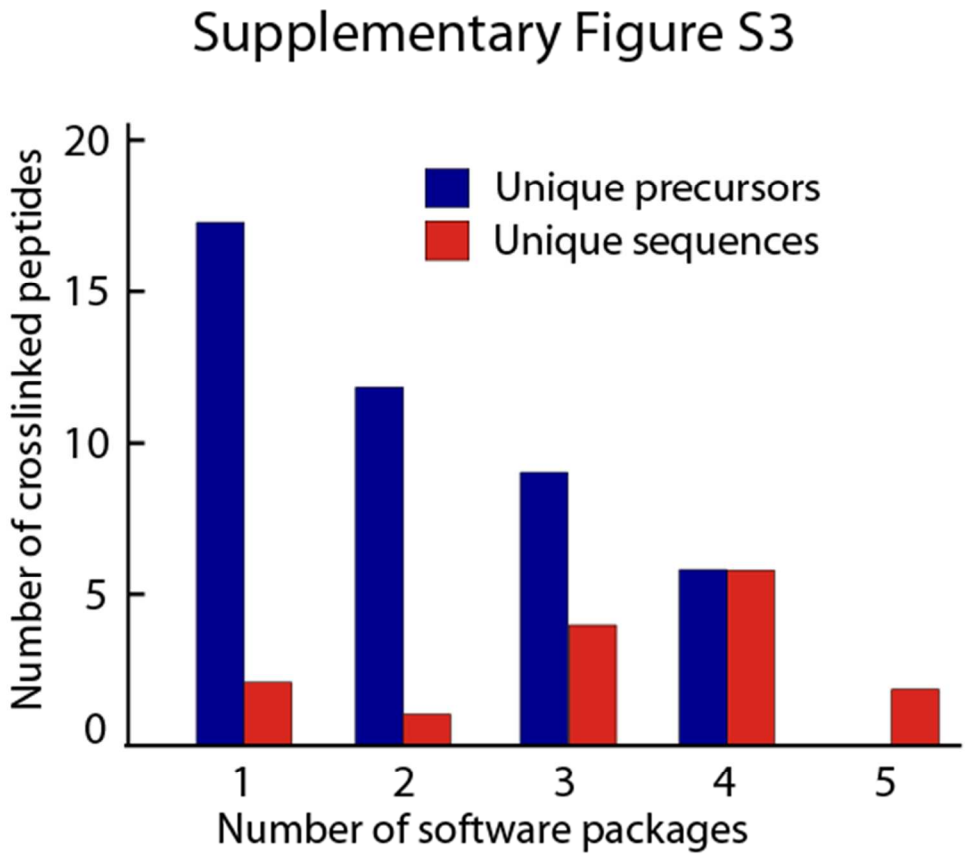


FIGURE S4. **False discovery rates of less than 1% were estimated for the intact red cell membrane and isolated membrane cytoskeleton samples**. FDR was normally calculated by counting the number of decoy crosslinked peptide hits. Adjusted FDR took into account the inherent bias between reverse-reverse decoy crosslinked peptides and reverse-forward decoy crosslinked peptides as described in (1). Asterisks mark the location of GM scores corresponding to < 1% FDR that were used as cutoffs for crosslink identification. (A) Intact membrane. (B) Isolated membrane cytoskeleton.

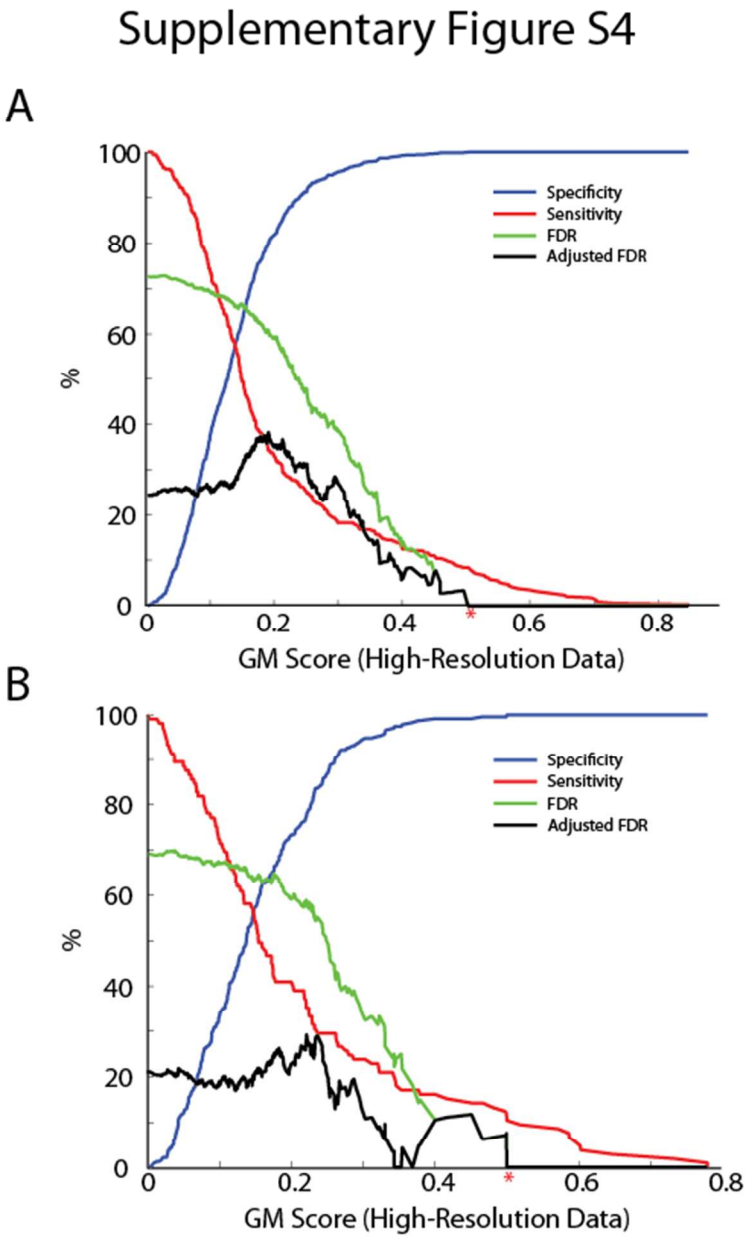


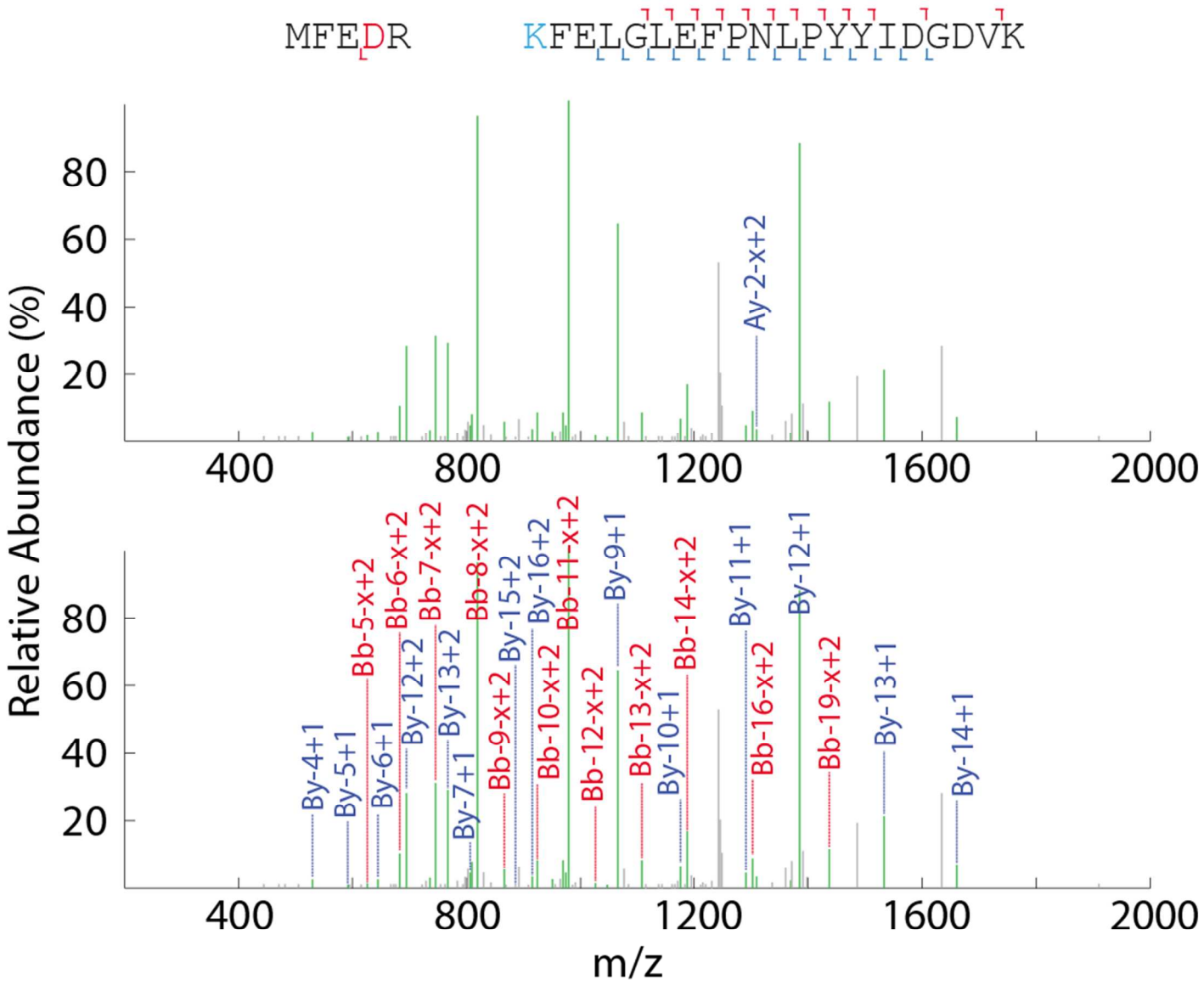
FIGURE S5. **Annotated MS/MS spectra for GST crosslinked peptides identified by ZXMiner whose Cα-Cα distances are significantly above 12 Å**. Peptide sequences along with location of identified b-ions and y-ions are at the top of each spectrum where red marks indicate ions that also contain the crosslinked site and intact second peptide and blue marks are regular b-ions and y-ions. Peaks colored in green are those that matched to theoretical ions. B-ions and y-ions are annotated in the spectrum with red and blue labels, respectively. Only major ions without neutral losses are shown. The top panel shows the contribution from the first peptide, and the bottom panel shows the contribution from the second peptide. Ion naming convention is as follow:

1. [Peptide][Ion Type]-[Ion Index]+[Charge] for regular ions. For example, Ab-10+2 represents a b-10 ion from peptide A (the first peptide) with charge state of +2.

2. [Peptide][Ion Type]-[Ion Index]-x+[Charge] for crosslinked ions. For example, Bb-10-x+4 represents a b-10 ion from peptide B (the second peptide) with charge state of +4 that also contains the crosslinked site and intact peptide A (the first peptide).

# indicates oxidized Met. } indicates the protein C-terminus.

A. Peptide: MFEDR-KFELGLEFPNLPYYIDGDVK, Charge State: +3, m/z: 1012.5001, GM Score: 0.52.



B. Peptide: YIAWPLQGWQATFGGGDHPPK}-VDFLSKLPEM#LK, Charge State: +3, m/z: 1248.3078, GM Score: 0.41.

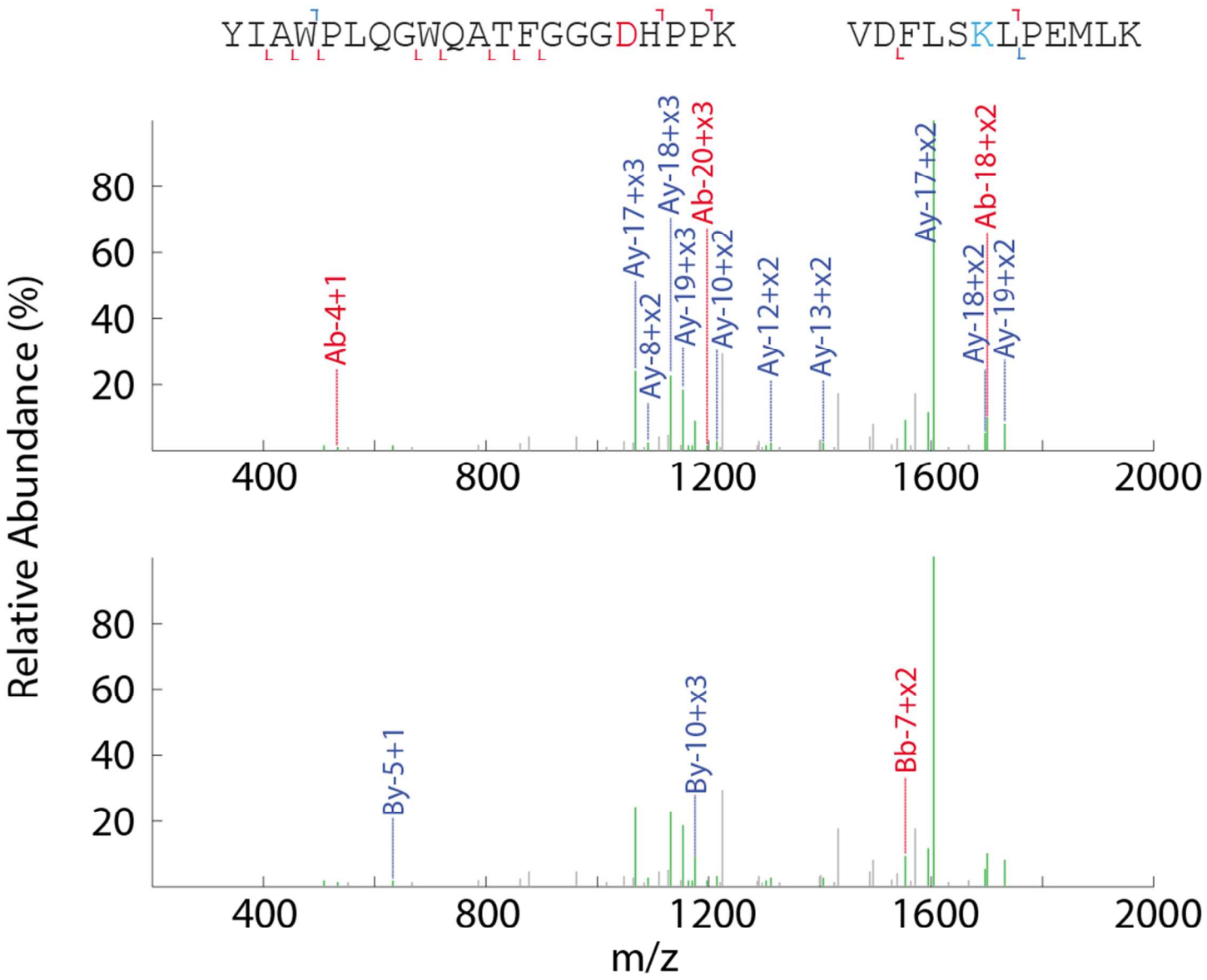


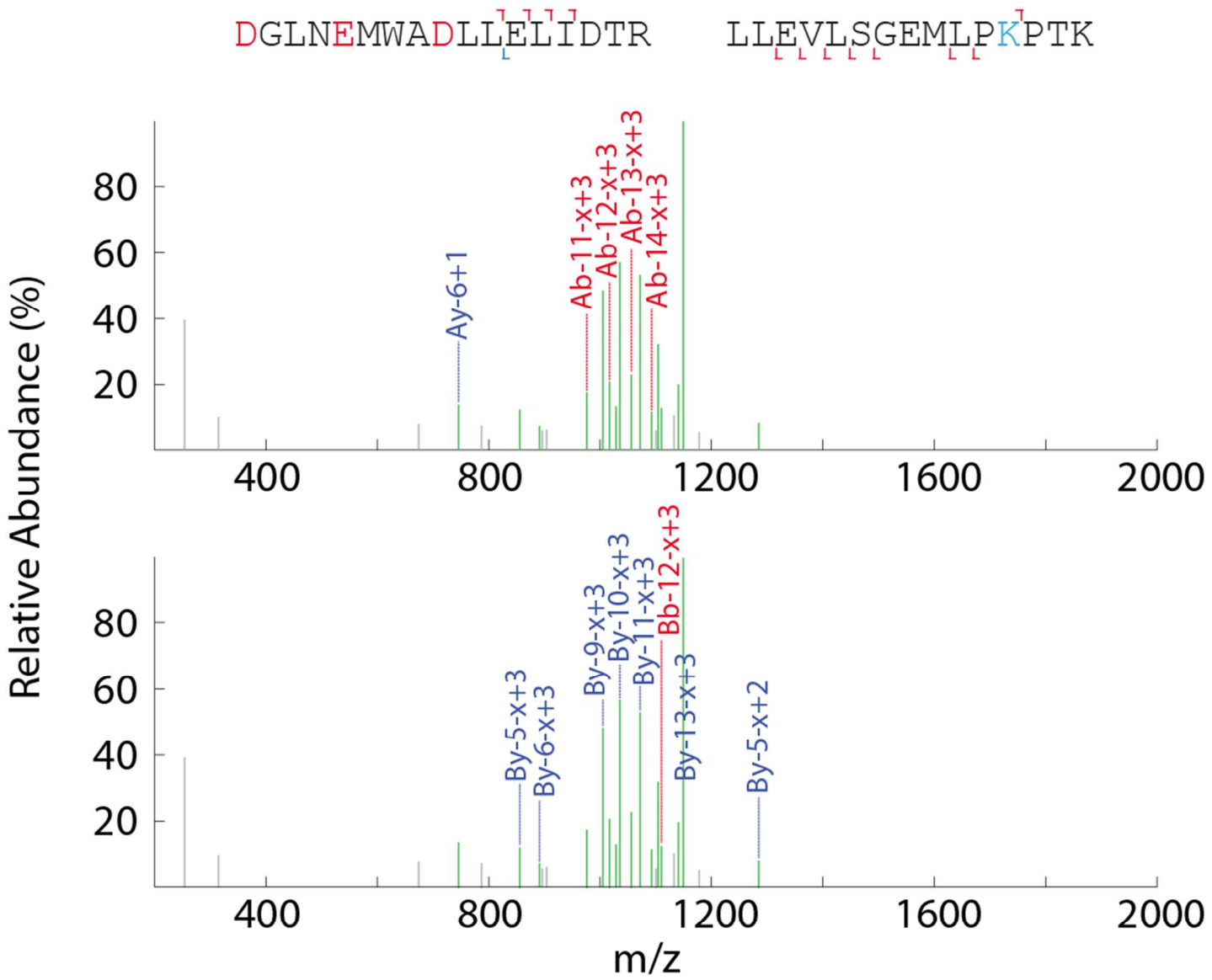
FIGURE S6. **Annotated MS/MS spectra for long-range crosslinked peptides identified by ZXMiner in the purified spectrin heterodimer**. Peptide sequences along with location of identified b-ions and y-ions are at the top of each spectrum where red marks indicate ions that also contain the crosslinked site and intact second peptide and blue marks are regular b-ions and y-ions. Peaks colored in green are those that matched to theoretical ions. B-ions and y-ions are annotated in the spectrum with red and blue labels, respectively. Only major ions without neutral losses are shown. The top panel shows the contribution from the first peptide, and the bottom panel shows the contribution from the second peptide. Ion naming convention is as follow:

1. [Peptide][Ion Type]-[Ion Index]+[Charge] for regular ions. For example, Ab-10+2 represents a b-10 ion from peptide A (the first peptide) with charge state of +2.

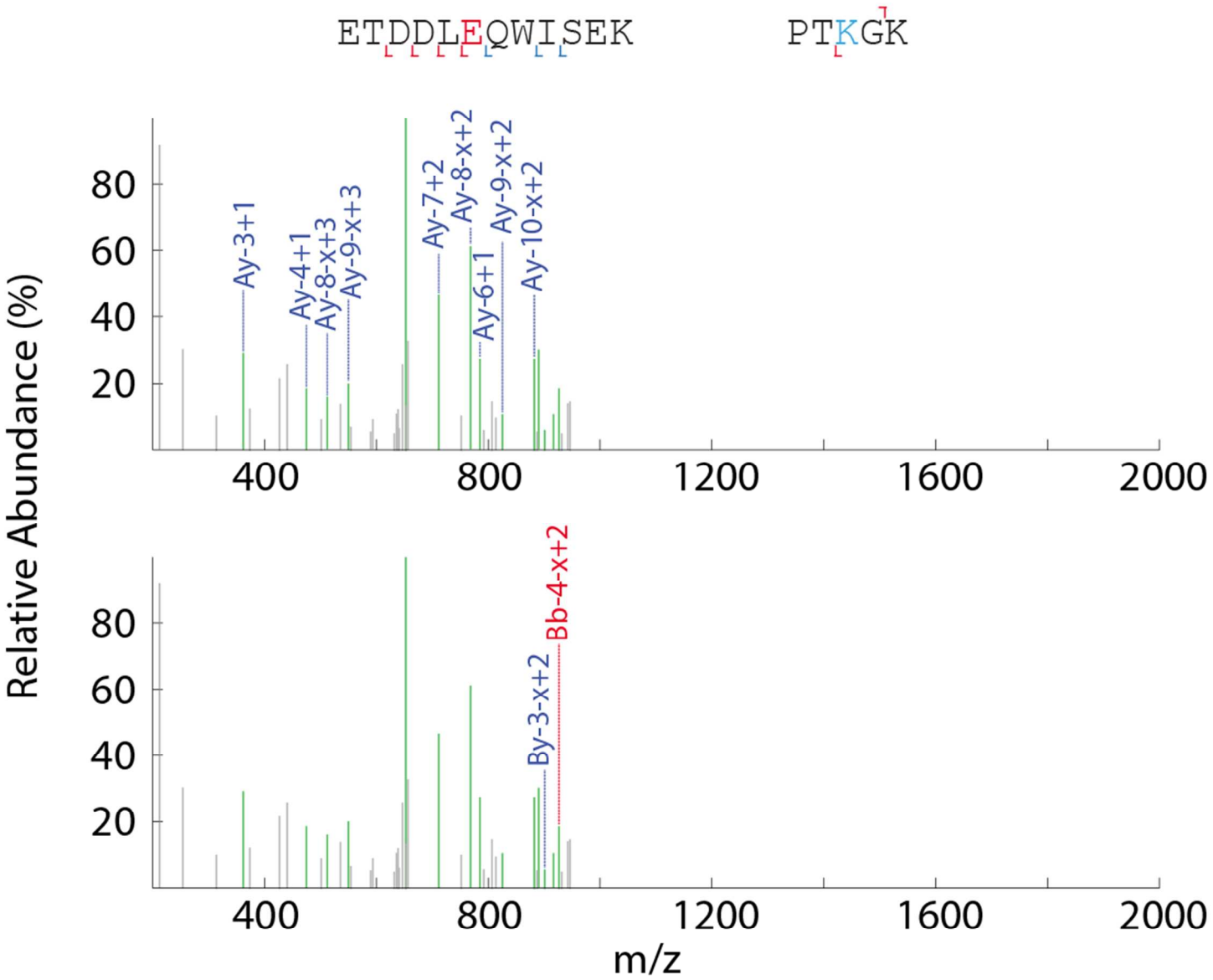
2. [Peptide][Ion Type]-[Ion Index]-x+[Charge] for crosslinked ions. For example, Bb-10-x+4 represents a b-10 ion from peptide B (the second peptide) with charge state of +4 that also contains the crosslinked site and intact peptide A (the first peptide).

# indicates oxidized Met.

A. Peptide: DGLNEM#WADLLELIDTR-LLEVLSGEM#LPKPTK, Charge State: +4, m/z: 918.7282, GM Score: 0.46.



B. Peptide: ETDDLEQWISEK-PTKGK, Charge State: +3, m/z: 668.6688, GM Score: 0.41.



C. Peptide: LSESHPDATEDLQR-FTEGKGYQPCDPQVIQDR, Charge State: +5, m/z: 744.1507, GM Score: 0.41.