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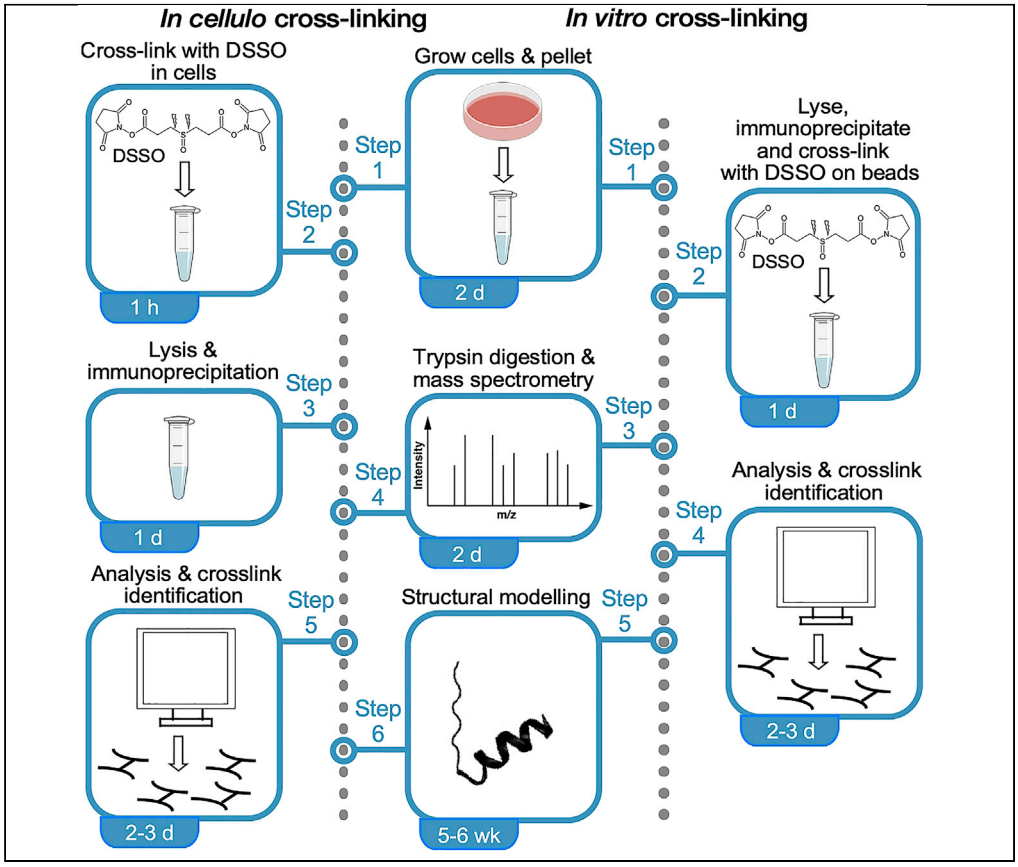
An optimized protocol for *in vitro* and *in cellulo* structural determination of the multi-tRNA synthetase complex by cross-linking mass spectrometry



Despite recent advances in structural determination of individual proteins, elucidating the 3-dimensional architecture of large, multiprotein complexes remains challenging, partly because of issues related to structural integrity during purification. Here, we describe a protocol to determine the 3-dimensional architecture of the 11-constituent, multi-tRNA synthetase complex (MSC) using chemical cross-linking coupled with mass-spectrometry (XL-MS). The protocol does not require purification and is broadly applicable, facilitating determination of native structures in cell lysates and in non-disrupted cells as well as in purified complexes.

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Highlights

Determines *in vitro* and *in cellulo* structures of multi-protein complexes

Facilitates analysis of multi-protein-complex architecture without purification

Reveals spatial relationships of disordered domains

Refines structures derived from X-ray crystallography which may be distorted by packing

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An optimized protocol for *in vitro* and *in cellulo* structural determination of the multi-tRNA synthetase complex by cross-linking mass spectrometry

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SUMMARY

Despite recent advances in structural determination of individual proteins, elucidating the 3-dimensional architecture of large, multiprotein complexes remains challenging, partly because of issues related to structural integrity during purification. Here, we describe a protocol to determine the 3-dimensional architecture of the 11-constituent, multi-tRNA synthetase complex (MSC) using chemical cross-linking coupled with mass-spectrometry (XL-MS). The protocol does not require purification and is broadly applicable, facilitating determination of native structures in cell lysates and in non-disrupted cells as well as in purified complexes.

For complete details on the use and execution of this protocol, please refer to Khan et al. (2020).

BEFORE YOU BEGIN

Principal technologies used to determine protein structures include X-ray crystallography (XRD), cryo-electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR). These approaches are challenged by the requirement for purification, instability of proteins and complexes during purification, low resolution of diffracting crystals, masses too small or too large for the specific technology, among other obstacles. Cross-linking mass-spectrometry (XL-MS) is a relatively recent addition to the armamentarium for protein structure elucidation. The technique combines chemical cross-linking of proteins and complexes followed by mass spectrometric analysis, revealing intra- and inter-protein cross-links indicative of proximate residues and domains. This "interaction" network can be used to model conformational states of protein and protein complexes (Mintseris and Gygi, 2020; Chavez et al., 2018). Disuccinimidyl sulfoxide (DSSO) is a MS-cleavable cross-linker, developed a decade ago, and cross-links Lys residues in peptides separated by about 10–27 Å (Sinz, 2017; Iacobucci et al., 2019). Cross-linkers with altered amino acid specificity, e.g., Cys or hydroxy-containing residues, or altered backbone length, can expand the coverage. The use of MS-cleavable cross-linkers facilitates the unambiguous determination of domain partners by improving specificity and reducing "false positives", particularly in experiments where interacting partners are unknown. The advantages of XL-MS have permitted resolution of an array of refractory multi-protein complexes (Kao et al., 2011; Gutierrez et al., 2016, 2018). XL-MS is often used to resolve structures of purified proteins or multi-protein complexes; less common are applications of XL-MS for resolution

of structures in complex mixtures such as cell lysates or intact cells and tissues (Chavez et al., 2013; Weisbrod et al., 2013). Here, we describe in detail an XL-MS-based protocol that we have developed to elucidate the 3-dimensional architecture of the multi-tRNA synthetase complex (MSC) using the Lys cross-linker DSSO in conjunction with molecular modeling. The MSC is a large, heteromeric complex consisting of nine tRNA synthetases and three auxiliary proteins (in eleven distinct polypeptides) with a combined mass of ~1.2 MDa. We used the HEK293T cell-line in our experiments; however, the described method is general and can be adapted to analyze the structure of individual proteins and multi-protein complexes in diverse systems.

Before initiating the experiment, prepare the solutions listed in the materials and equipment section. Cool the cell lysis buffer to 4°C and add protease inhibitor before cell lysis. Filter solutions with 0.22 μm filters. All steps are performed at 4°C unless otherwise described.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Normal rabbit IgG [Dilution (1:100)]	Cell Signaling Technology	2729S
EPRS1, rabbit polyclonal [Dilution (1:100)]	BioSynthesis	N/A
Chemicals, peptides, and recombinant proteins		
1 M Tris-HCl (pH-7.5)	Boston BioProducts	BBT-75
1 M Tris-HCl (pH-7.8)	Boston BioProducts	BBT-78
5 M NaCl	Thermo Fisher Scientific	Cat#24740011
Triton X-100	Sigma-Aldrich	T8787
Bovine serum albumin	Sigma-Aldrich	A8806
Fetal Bovine Serum	Gemini	Cat#100-106
1 M HEPES (pH 7.8)	Boston BioProducts	BBH-78
1 M HEPES (pH 8.0)	Boston BioProducts	BBH-80
1 M $MgCl_2$	Alfa Aesar	J61014
Dithiothreitol	Sigma-Aldrich	Cat#10708984001
Disuccinimidyl sulfoxide (DSSO)	Thermo Fisher Scientific	A33545
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418
Ammonium bicarbonate	Sigma-Aldrich	A6141
Protease inhibitor	Thermo Fisher Scientific	78429
Acetic acid	Fisher Chemical	A113-50
Formic acid	Fisher Chemical	A117-50
Acetonitrile	Fisher Chemical	A955-4
Sequencing Grade Modified Trypsin	Promega	V5111
HBSS	Corning	T55-022-PB
Trypsin for cell culture	Caisson	T019
EDTA	Sigma-Aldrich	E6511
Sodium bicarbonate	Sigma-Aldrich	S6014
Phenol red	Caisson	P024
Penicillin	Sigma-Aldrich	P3032-100MU
Streptomycin	Gibco	11860-038
L-Glutamine	Caisson	G010
DMEM base powder	Caisson	DMP08
PBS base powder	Gibco	21600-044
12N HCl	Fisher Scientific	A144-212
Experimental models: Cell lines		
Human: HEK293T	ATCC	CRL-3216
Critical commercial assays		
Protein A/G magnetic beads	Thermo Fisher Scientific	Cat#88803

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
C18 spin columns	Thermo Fisher Scientific	Cat#89873
Mini Kleenpak	Pall	KA02DJLP2S
Supor DCF	Pall	CFS91SSPZK
Acropak 500	Pall	Cat#12995
MILLEX GP	Millipore	SLGP033RS
Software and algorithms		
Proteome Discoverer	Thermo Fisher Scientific	https://planetorbitrap.com/proteome-discoverer
XlinkX	Thermo Fisher Scientific	https://www.hecklab.com/software/xlinkx/
PyMol	Schrödinger	https://pymol.org/2/
Swiss-Model	Protein Structure and Bioinformatics group	https://swissmodel.expasy.org/
PatchDock	Wolfson Lab, Tel Aviv University	https://bioinfo3d.cs.tau.ac.il/PatchDock/
Other		
Microcentrifuge	Eppendorf	5424R
Minicentrifuge	Benchmark	C1008
Thermomixer	Eppendorf	Cat#4376600
DynaMag-2 magnet	Thermo Fisher Scientific	12321D
Plate reader	Molecular Devices	SpectraMax 190
Mass spectrometer	Thermo Fisher Scientific	Fusion Lumos
NanoHPLC Pump: NCS-3500RS Nano ProFlow	Thermo Fisher Scientific	5041.0010A
NanoHPLC Autosampler: WPS-3000TPL RS	Thermo Fisher Scientific	5826.0020
NanoHPLC Degasser: SRD-3400	Thermo Fisher Scientific	5035.9245
Trapping column	Thermo Fisher Scientific	Cat#164564
Analytical column	Thermo Fisher Scientific	Cat#164940

MATERIALS AND EQUIPMENT

Composition of buffers:

Cell lysis buffer (100 mL)	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	100 mM	10 mL
5 M NaCl	150 mM	3 mL
Triton X-100	1%	1 mL

Add double-distilled (dd) H_2O to 100 mL and store at 4°C for up to 3–6 months.
Add freshly made complete protease inhibitor cocktail to a 1× final concentration before use.

Trypsin-EDTA (100 mL)	Final concentration	Amount
HBSS	1×	951 mg
Trypsin for cell culture	0.05%	50 mg
EDTA	0.53 mM	17.3 mg
$NaHCO_3$	4.16 mM	35 mg
Phenol red	0.027 mM	1.1 mg

Add ddH_2O to 100 mL. Bring the pH to 7.0 by bubbling the solution with CO_2 gas. Filter the final solution using 0.1 micron Mini Kleenpak filter. Store at 4°C for up to 2–3 weeks.

Phosphate-buffered saline (1 L)	Final concentration	Amount
PBS base powder	1×	9.55 g

Add ddH_2O to 1 L. Bring the pH to 7.0 with 12N HCl. Filter the final solution using 0.22 micron Acropak 500 filter. Store at 25°C for up to 2 months.

Penicillin-streptomycin solution, 10,000 U/mL (100 mL)	Final concentration	Amount
Penicillin	10,000 U/mL	Depends on lot potency
Streptomycin	10,000 μg/mL	Depends on lot potency
NaCl	0.85%	0.85g

Add ddH_2O to 100 mL. The pH is ~5.5. Filter the final solution using 0.1 micron Mini Kleenpak filter. Store at −20°C for up to 1 year. Thaw at 37°C water bath just before supplementing DMEM media.

L-glutamine solution (100 mL)	Final concentration	Amount
L-glutamine	200 mM	2.923g
NaCl	0.85%	0.85g

Add ddH_2O to 100 mL. The pH is ~5.5. Filter the final solution using 0.1 micron Mini Kleenpak filter. Store at −20°C for up to 1 year. Thaw at 37°C water bath just before supplementing DMEM media.

Dulbecco's modified Eagle's Medium/DMEM (1 L)	Final concentration	Amount
Base Media	1×	11.49 g
Sodium bicarbonate	44 mM	3.7 g
Penicillin-streptomycin solution	100 U/mL	10 mL
L-glutamine	2 mM	10 mL
FBS	10%	100 mL

Add ddH_2O to 1 L. Bring the pH to 7.0 by bubbling the solution with CO_2 gas. Filter the final solution using 0.1 micron Supor DCF filter. Add FBS, penicillin-streptomycin and L-glutamine solution once you open a new bottle of DMEM. Store at 4°C for up to 4 weeks.

Pre-blocking buffer (10 mL)	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	100 mM	1 mL
5 M NaCl	150 mM	0.3 mL
Triton X-100	1%	0.11 mL
Bovine serum albumin (BSA)	0.02%	2 mg

Add ddH_2O to 10 mL. Prepare just before use as BSA is highly susceptible to microbial contamination. Add complete protease inhibitor cocktail to a 1× final concentration prior to use.

Hypotonic buffer (100 mL)	Final concentration	Amount
1 M HEPES (pH 8.0)	10 mM	1 mL
5 M NaCl	10 mM	2 mL
1 M $MgCl_2$	1 mM	0.1 mL
Dithiothreitol (DTT)	0.5 mM	0.05 mL

Add ddH_2O to 100 mL. Store at 4°C for up to 3–6 months.
Add DTT and complete protease inhibitor cocktail to a 1× final concentration just before use.

Wash buffer (100 mL)	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	50 mM	5 mL
5 M NaCl	150 mM	3 mL
Triton X-100	0.5%	0.5 mL

Add ddH_2O to 100 mL. Store at 4°C for up to 3–6 months.

Cross-linking buffer (100 mL)	Final concentration	Amount
1 M HEPES (pH 7.8)	20 mM	2 mL
5 M NaCl	150 mM	3 mL

Add ddH_2O to 100 mL. Store at 4°C for up to 3–6 months.

Quenching buffer	Final concentration	Amount
1 M Tris-HCl (pH 7.8)	20 mM	As needed based on bead resuspension volume
Can store at 4°C for 3–6 months.		

Crosslinker:

Prepare working stock (50 mM) of DSSO by dissolving in DMSO.

STEP-BY-STEP METHOD DETAILS

Cell culture and preparation of pellet

Timing: 2 days before start of experiment

1. Seed two 150-mm dishes with 5 × 10^6 HEK293T cells (between passages 2–10). Culture until about 90% confluent (~48 h) in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, L-glutamine (2 mM) and sodium bicarbonate (44 mM) in incubator with 5% CO_2 at 37°C.
2. Aspirate medium and wash adherent cells twice with phosphate-buffered saline (PBS). Detach cells with 3 mL of trypsin-EDTA, 5 min at 37°C.
3. Combine cells from both dishes, and resuspend in 10 mL of DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, L-glutamine (2 mM), and sodium bicarbonate (44 mM) in a 15 mL centrifuge tube, and pellet by centrifuging at 1,000×*g* for 5 min.
4. Remove medium and wash cells twice with ice-cold PBS, and re-pellet cells at 1,000×*g* for 5 min.

Note: For *in situ* cross-linking of live cells (i.e., not lysates) proceed to step 24 (i.e., skip steps 5–23).

⚠ CRITICAL: The cells can be snap-frozen at this stage and stored at −80°C. Altered interaction of MSC constituents was not observed by freezing. However, the stability of other protein-complexes may be affected by the freeze-thaw process, and should be determined by immunoprecipitation experiments before proceeding to cross-linking.

Lysis of cells and preparation of lysate

Timing: 2 h

Note: Protein A/G magnetic beads (Pierce) should be blocked in pre-blocking buffer for 1 h during cell lysis procedure in readiness for pre-clearing step when cell lysates are being prepared.

⚠ CRITICAL: Use ice-cold lysis buffer and add protease inhibitor just before cell lysis.

5. Add protease inhibitor to 1× final concentration in lysis buffer.
6. Add a 1.4 mL volume of lysis buffer to the cell pellet and mix gently by trituration until particulates are solubilized to homogeneity.
7. Transfer lysate to a 2-mL eppendorf tube and mix it in a rotator end-to-end for 30 min at 4°C.
8. Centrifuge at 21,000 *g* for 20 min at 4°C.
9. Transfer by pipette the clarified cell lysate into a 2 mL eppendorf tube.
10. Estimate lysate protein concentration by BCA or Bradford assay.
11. Divide lysate equally (generally between 2-3 mg protein) into two 1.5 mL eppendorf tubes.
12. Add a 30 µL volume of pre-blocked Protein A/G magnetic beads to each tube and mix in end-to-end rotator for 1 h at 4°C.

13. Separate lysate from magnetic beads using a DynaMag-2 magnet and transfer to fresh 1.5 mL eppendorf tube. The lysate is ready for immunoaffinity pulldown.

Immunoaffinity pulldown using antibody targeting bait protein, and chemical cross-linking of bait and associated proteins

Timing: 1 day

14. Add target-specific rabbit polyclonal antibody in one tube and IgG control antibody in the other, and mix in an end-to-end rotator for 4 h at 4°C. In our experiments we used an antibody against human glutamyl-prolyl tRNA synthetase (EPRS1) custom generated using the human EPRS1 linker domain (Leu753 to Thr956).
15. Add a 60 μL volume of pre-blocked Protein A/G magnetic beads suspended in lysis buffer to each tube, and mix for 12–16 h in an end-to-end rotator at 4°C.

Note: There are multiple ways to do the pull-down. The antibody can be first immobilized on Protein A/G magnetic beads to be added directly to the cell lysate followed by overnight incubation. Alternatively, the antibody can be incubated overnight with the cell lysate, and Protein A/G magnetic beads added the next morning for 2–4 h incubation in an end-to-end rotator.

⚠ CRITICAL: The antibody-to-lysate ratio will differ for every bait protein, and should be standardized before proceeding with the pull-down.

16. Next day, the beads are collected using a DynaMag-2 magnet and the lysate carefully withdrawn by pipet without disturbing the beads.
17. The beads are washed with a 1 mL volume of ice-cold wash buffer for 15 min on an end-to-end rotator at 4°C.
18. Collect beads again using DynaMag-2 magnet and repeat the wash step three more times.
19. After removing the final wash buffer, wash the beads again with 1 mL of cross-linking buffer two times to completely remove residual Tris buffer.

⚠ CRITICAL: Care must be taken to completely remove any trace of Tris buffer from the beads as the amine-containing buffer interferes with the DSSO cross-linking reaction, and in fact, is used to quench the reaction as described below. Final pH of the cross-linking buffer should be between pH 7.8–8.0.

Note: During the final washing step with cross-linking buffer, prepare a 50 mM DSSO solution by dissolving 0.5 mg of DSSO in 26 μL of DMSO, followed by a brief vortex to dissolve. For *in cellulo* cross-linking skip steps (20) to (23).

20. After the final wash, resuspend beads in a 400 μL volume of cross-linking buffer and add an 8 μL volume of the 50 mM DSSO solution to yield a final concentration of 1 mM. Discard leftover DSSO solution.
21. Vortex beads gently to resuspend and mix for 45 min at 25°C in a thermomixer.

⚠ CRITICAL: The beads tend to settle in the thermomixer. For efficient cross-linking, resuspend beads by gentle vortexing every 15 min.

22. Stop the reaction by addition of a 8 μL volume of quenching buffer (20 mM final concentration) for 30 min at 25°C in thermomixer.
23. Collect cross-linked beads using a DynaMag-2 magnet and proceed to on-bead tryptic digestion for mass spectrometry (step 28).

Alternate procedure for cross-linking in live cells

Timing: 1 day

24. Resuspend pelleted HEK293T cells in a 1.88 mL volume of hypotonic buffer containing protease inhibitor cocktail.
25. Add a 120 μL volume of DSSO (50 mM in DMSO; prepared by dissolving 2.5 mg DSSO in 130 μL volume of DMSO) to yield a final concentration of 3 mM, and mix for 1 h in an end-to-end rotator at 4°C. Discard leftover DSSO solution.

⚠ CRITICAL: Incubation time can vary based on cell type and the protein or complex of interest. This should be standardized on a case-by-case basis by progressively increasing the incubation time and scoring for new-cross links.

26. The cross-linking reaction is stopped by addition of a 40 μL volume of quenching buffer (1 mM Tris-HCl, pH 7.8 to final concentration of 20 mM) for 30 min on an end-to-end rotator.
27. Return to steps (5) through (19). After completion of step (19) proceed to step (28).

Trypsin digestion and sample preparation for mass spectrometry

Timing: 2 days

28. Resuspend beads in a 10 μL volume of 100 mM ammonium bicarbonate containing 100 ng trypsin (10 ng/μL).
29. Vortex samples for 15 s every 2–3 min for 15 min at room temperature (~25°C).
30. Continue digestion for 12–16 h at 37°C in thermomixer.
31. Next morning, add a second aliquot of 100 ng trypsin in 100 mM ammonium bicarbonate, and incubate in thermomixer at 37°C for 4 h.
32. Collect supernatant using a DynaMag-2 magnet, and dilute with formic acid (5% v/v, final concentration).
33. Remove salts from supernatants with PepClean C-18 spin columns, and dry sample completely with a vacuum concentrator.
34. Reconstitute dried samples in a 30 μL volume of 1% acetic acid.

Mass spectrometry and identification of proximate cross-linked peptides

Timing: 1 day

35. Perform LC-MS experiments using an Orbitrap Fusion Lumos Tribrid MS instrument coupled to an Ultimate 3000 UHPLC system equipped with a 2-cm reverse-phase C18 trapping column and a 15-cm reverse-phase C18 analytical column.
36. Load a 5 μL volume of digested peptides (1–2 μg protein) onto the trapping column with solvent A (0.1% formic acid), at a flow rate of 10 μL/min for 5 min. After desalting, elute the peptide onto the analytical column and fractionate for 140 min using a gradient of solvent A/solvent B (80% acetonitrile/20% water/0.1% formic acid) at a flow rate of 0.3 μL/min.

Gradient:

Time (min)	% Solvent A	% Solvent B
0	98	2
5	98	2
110	65	35

(*Continued on next page*)

Continued		
Time (min)	% Solvent A	% Solvent B
120	10	90
127	10	90
129	98	2
140	98	2

37. Perform three LC-MS/MS experiments on the cross-linked samples i.e., a data-dependent acquisition (DDA), MS2-MS3, and electron-transfer and higher-energy collision dissociation (EThcD).

Note: See Table 1 for parameters used in LC-MS/MS experiments.

38. For the DDA method, switch between MS1 scans for molecular weight measurement and MS/MS experiments for peptide sequencing.
39. For the MS2-MS3 experiments, perform low energy MS2 experiments on ions with charges between 4 and 8. Perform MS3 experiments on ions with a mass difference of 31.9721 Da, i.e., the mass difference between the alkene and thiol forms of DSSO cross-linked peptides.
40. For EthcD experiments, perform two MS2 experiments, EThcD and low-energy collision-induced dissociation (CID) experiments on ions with charges between 4 and 8.

Data and error analysis for mass spectrometry

Timing: 1–2 days

41. Perform data analysis in Proteome Discoverer 2.4.

Note: See Figure 1 for processing and workflow for analysis of XL-MS datasets using Proteome Discoverer 2.4.

42. For analysis of the DDA dataset, search against a species-specific sequence database such as human SwissProtKB (see parameters in Table 1).
43. For DDA searches, export protein identifications for all proteins identified by at least 2 peptides at a peptide and protein false discovery rate (FDR) of 1%.

Note: Peptide FDR rates are estimated using the Percolator algorithm, a semi-supervised machine learning approach that compares peptide scores in searches performed against the forward and reverse (decoy) databases. Only high-confidence peptides are considered.

44. For analysis of XL-MS datasets, use the XlinkX node bundled into Proteome Discoverer 2.4 (see parameters in Table 2) to search against the database generated in the DDA analysis.

Note: FDR is estimated using XlinkX validator node. Only consider high-confidence peptides.

45. Cross-links identified in the MS2-MS3 analysis are interrogated to ensure that 3 or more of the 4 expected peptides are fragmented, and the spectra for these peptides contain sequence-specific ions consistent with the identified peptides.
46. Cross-links identified from the EthcD analysis are interrogated to ensure that sequence-specific ions consistent with both cross-linked peptides are identified in the spectrum.

⚠ CRITICAL: Prior to running cross-linked samples, perform an instrument QC to check chromatographic response, instrument response, and mass calibration.

Table 1. Parameters for LC-MS/MS analysis

	DDA	MS2-MS3	EthcD
LC parameters			
Mobile phase A	0.1% Formic acid	0.1% Formic acid	0.1% Formic acid
Mobile phase B	80% ACN, 0.1% formic acid	80% ACN, 0.1% formic acid	80% ACN, 0.1% formic acid
Trapping column	Dionex (2 cm × 100 μm id) Acclaim Pepmap C18, 5 μm, 100 Å	Dionex (2 cm × 100 μm id) Acclaim Pepmap C18, 5 μm, 100 Å	Dionex (2 cm × 100 μm id) Acclaim Pepmap C18, 5 μm, 100 Å
Analytical column	Dionex (25 cm × 75 μm id) Acclaim Pepmap C18, 2 μm, 100 Å	Dionex (25 cm × 75 μm id) Acclaim Pepmap C18, 2 μm, 100 Å	Dionex (25 cm × 75 μm id) Acclaim Pepmap C18, 2 μm, 100 Å
Flow rate: trapping, loading time	5 μL/min, 5 min	5 μL/min, 5 min	5 μL/min, 5 min
Flow rate: analytical	0.3 μL/min	0.3 μL/min	0.3 μL/min
Column oven temperature	36°C	36°C	36°C
Gradient	0 min, 2% B 5 min, 2% B 110 min, 35% B 120 min, 90% B 127 min, 90% B 129 min, 2% B 140 min, 2% B	0 min, 2% B 5 min, 2% B 110 min, 35% B 120 min, 90% B 127 min, 90% B 129 min, 2% B 140 min, 2% B	0 min, 2% B 5 min, 2%B 110 min, 35% B 120 min, 90% B 127 min, 90% B 129 min, 2% B 140 min, 2% B
MS1 parameters			
Spray voltage	2.3 kV	2.3 kV	2.3 kV
MS1: detection	Orbitrap	Orbitrap	Orbitrap
MS1: resolution	120,000	60,000	60,000
MS1: scan range	350–1,500 Da	350–1,500 Da	350–1,500 Da
MS1: mode	Profile	Profile	Profile
MS1: AGC target	4.0 e5	4.0 e5	4.0 e5
Maximum injection time	50 msec	50 msec	50 msec
MS2: CID parameters			
MS2: charge states	2–7	4–8	4–8
MS2: cycle time	3 s	5 s	5 s
MS2: isolation window	0.7	1.6 Da	1.6 Da
MS2 fragmentation method	CID	CID	CID
MS2: collision energy	35%	25%	25%
MS2: ETD activation	False	False	False
MS2: detection	Ion trap	Orbitrap	Orbitrap
MS2: resolution	Unit	30,000	30,000
MS2: AGC target	3.0 e3	5.0 e4	5.0 e4
MS2: mode	Centroid	Centroid	Profile
Maximum injection time	300 msec	100 msec	100 msec
Dynamic exclusion	Enabled	Enabled	Enabled
Exclude after n times	1	1	1
Exclusion duration	60 s	60 s	60 s
MS2: Ethcd parameters			
MS2: charge States	–	–	4–10
MS2: cycle time	–	–	5 s
MS2: isolation window	–	–	1.6 Da
MS2 fragmentation method	–	–	ETD
MS2: ETD activation	–	–	True
ETD: collision energy	–	–	15%
MS2: detection	–	–	Orbitrap
MS2: resolution	–	–	30,000
MS2: AGC Target	–	–	1.0 e5
MS2: mode	–	–	Centroid
Maximum injection time	–	–	120 msec
Dynamic exclusion	–	–	Enabled
Exclude after n times	–	–	1

(*Continued on next page*)

Table 1. *Continued*

	DDA	MS2-MS3	EthcD
Exclusion duration	–	–	60 s
MS3: parameters	DDA	MS2-MS3	EthcD
Target mass difference	–	31.9721 Da	–
Ions must be same charge	–	Yes	–
MS2: charge states	–	2–6	–
MS2: scans	–	4	–
MS2: isolation window	–	2.0 Da	–
MS2 fragmentation method	–	HCD	–
MS2: collision energy	–	30%	–
MS2: ETD activation	–	False	–
MS2: detection	–	Ion trap	–
MS2: resolution	–	Nominal	–
MS2: AGC target	–	2.0 e4	–
MS2: mode	–	Centroid	–
Maximum injection time	–	150 s	–

Cross-link validation and modeling individual complex constituents

Timing: 3 weeks

47. Our XL-MS-based analysis of the MSC revealed a total of 137 cross-links, including both intra- and inter-protein cross-links (Figure 2). *In vitro* and *in cellulo* analyses yielded 131 and 57 cross-links, respectively, consistent with a substantially greater efficiency of the former (Tables S1 and S2). However, the overlap between the groups was high as 51 of 57 cross-links detected *in cellulo* were also present in the *in vitro* analysis. Importantly, no inconsistencies, e.g., steric conflicts, were introduced by cross-links detected by either approach.
48. For cross-links in constituents or sub-complexes of known structure, use reported crystal or NMR structure from Protein Data Bank (PDB) to validate newly obtained intra- and inter-protein cross-links (e.g., in our experiment, EPRS1 and KARS1-AIMP2, respectively (Figure 3).
49. To build structures for proteins where homologous structures are available for non-human species only, perform homology modeling using SWISS-MODEL program.
50. For individual constituents of unknown or incomplete structure, perform de novo modeling with SWISS-MODEL to build full-length structure. SWISS-MODEL uses a library of previously published structures to search for proteins with significant sequence similarity compared to the sequence of the modelled protein. Construct model from structurally conserved regions extracted from selected template structures; missing residues can be modeled de novo. Accommodate the distance restraints provided by intra-protein crosslinks during modeling.

Note: Detailed information regarding the modeling of individual constituents of the MSC has been reported (Khan et al., 2020).

Adjustment of reported structures to conform to cross-link data

Timing: 1–2 days

Note: Cross-links of Lys residues at distances between about 10 and 27 Å in crystal or NMR structures validate the approach and methodology. Occasionally, distances between cross-linked amino acids in published X-ray structures can substantially exceed the ~27 Å distance limit. The discrepancy between the X-ray and XL-MS data can result from low-confidence data in the crystal structure due to mobile domains, distortion of the structure due to high-density

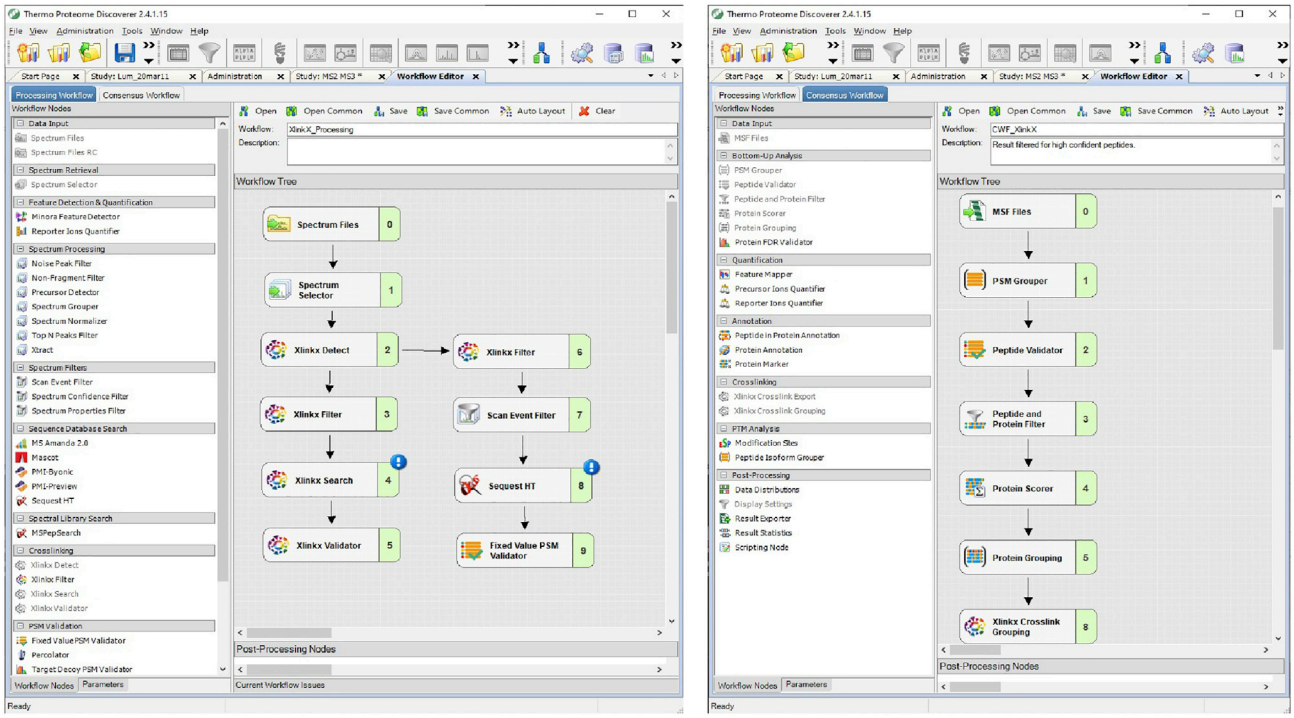

Figure 1. Workflow for processing and for analysis of XL-MS datasets using Proteome Discoverer 2.4

packing in the crystal, or altered conformation of the target when present under intracellular conditions, e.g., in a complex.

51. When sufficient justification is provided, e.g., by multiple discrepant cross-links, modify the conformation to constrain the lengths of these cross-links to less than ~27 Å (Figure 4). Biochemical activity data can provide support for the altered conformation, as was the case with the XL-MS-based structural modification of QARS1 (Ognjenovic et al., 2016; Khan et al., 2020).

⚠ CRITICAL: Dimers, particularly those in a head-to-tail conformation, can confound the analysis as apparent long cross-links in the monomeric crystal structure can be due to short cross-links spanning the dimer interface (Figure 5).

Modeling of the holo-MSC

Timing: 3 weeks

52. Using the protein-protein docking program PatchDock, assemble the MSC in a step-wise manner by one-at-a-time docking constituents or sub-complexes to the partially assembled complex retaining the constraints provided by inter-molecular cross-links. For example, the modified QARS1 model was docked with the pentameric AARS core (comprising EPRS1, MARS1, AIMP2, AIMP3, and DARS1) using the distant constraints obtained from QARS1-EPRS1 and QARS1-AIMP2 intermolecular cross-links.
53. Visualize model using the molecular visualization program PyMOL (see Figure 6 for example).

EXPECTED OUTCOMES

The current protocol is derived from scoring cross-links between MSC components pulled down from HEK293T cells using an antibody against one constituent, EPRS1. It identifies proximate Lys

Table 2. Parameters for proteome discoverer searches

LC parameters	DDA	MS2-MS3	EthcD
Database	Sequence-specific SwissProt	Generated from DDA experiment	Generated from DDA experiment
Precursor selection	Use MS1 precursor	Use MS (n-1) with parent precursor	Use MS (n-1) parent precursor
Acquisition strategy	Not applicable	MS2_MS3	MS2_MS2
Mass tolerance MS1	10 ppm	10 ppm	10 ppm
Mass tolerance MS1	0.5 Da (ITMS)	20 ppm (FTMS) 0.5 Da (ITMS)	20 ppm (FTMS)
Dynamic modifications	Oxidation (M) DSSO modification (K, 158.004 Da) Acetylation (Protein N-term)	Oxidation (M) DSSO modification (K, 158.004 Da) Acetylation (Protein N-term)	Oxidation (M) DSSO modification (K, 158.004 Da) Acetylation (Protein N-term)
Peptide FDR (Percolator)	0.01	–	–
Peptide FDR (Xlinkx Validator)	–	0.01	0.01
Additional criteria for identification	Two peptides per protein	High quality MS/MS spectra identified for 3 out of the 4 DSSO (alkene and thiol) peptides	Able to identify sequence specific ions consistent with both cross-linked peptides in the spectrum.

residues both within and between the protein constituents of the human MSC. Intra-protein cross-link data can append domains or refine individual protein structures, and can be combined with inter-protein cross-link data for modeling the entire complex.

LIMITATIONS

This protocol takes advantage of a well-characterized, highly specific antibody used extensively in our laboratory, and efficiently pulls down endogenous EPRS1. We have not tested this protocol using over-expressed epitope-tagged proteins. However, we do not anticipate substantial technical issues with this approach beyond the possibility that the tag interferes with protein-protein interactions. Because the MSC is a relatively stable complex, we could use stringent conditions for lysis and washing steps. Possibly, such stringent condition will disrupt weak or transient interactions in less stable complexes. We recommend initially using less stringent conditions for lysis and washing, and testing the presence of the bait and target proteins following immuno-pull down by western blot analysis before proceeding with XL-MS.

TROUBLESHOOTING

Problem 1

Low expression of target protein or complex (Check before starting the experiment).

Potential solution

Increase the amount of starting material

Increase bait protein expression by induction or transfection. If the complex contains two proteins, then co-express both components of the complex.

Problem 2

Absence of interacting proteins following pull-down (steps 5–18).

Potential solution

The absence of interacting proteins can be due to transient nature of the complex, or instability during lysis and pull-down. In this case, lysis conditions can be explored by varying the buffer,

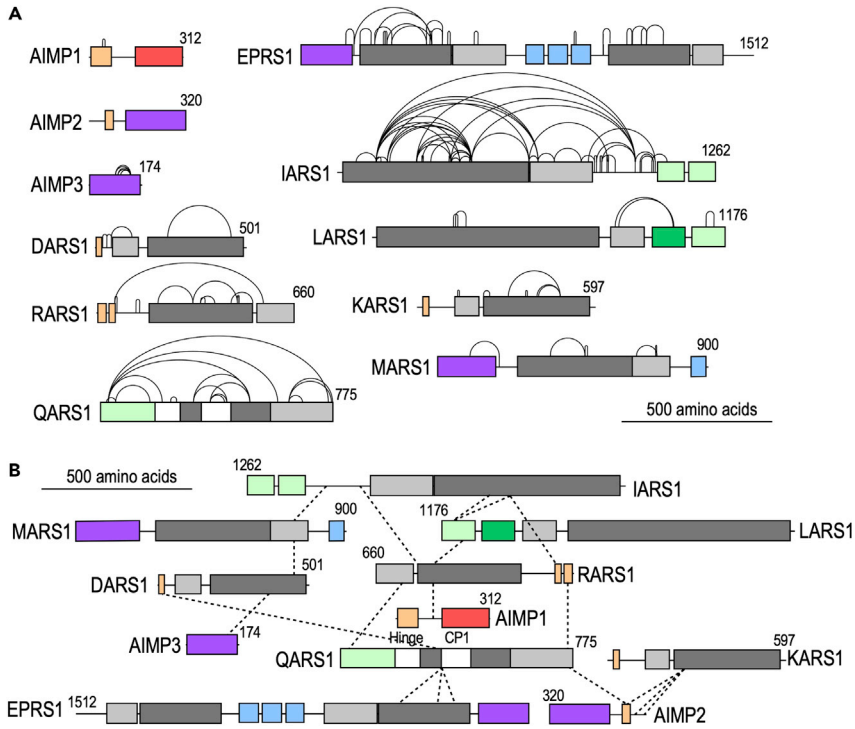

Figure 2. XL-MS-derived crosslinks for human MSC

(A) Intra-protein cross-links within constituents of the MSC (black curved lines).

(B) Inter-protein cross-links between constituents of the MSC (dashed lines).

salt, and detergent composition to determine the optimal lysis buffer for isolation of the complex. Also, use of a milder wash buffer under these conditions is recommended.

Problem 3

No cross-links are detected (steps 35–46).

Potential solution

This might be due to absence of accessible or proximate target residues, e.g., Lys in case of DSSO, in the bait or target proteins. In this case, MS-cleavable cross-linkers with alternative amino acid specificity, e.g., BMSO for Cys cross-links or DHSO for acidic amino acid cross-links can be considered (Gutierrez et al., 2016, 2018). In the case of poor accessibility of a residue, for example if the reactive amine group is not near the surface, increasing the concentration of the cross-linker or increasing the incubation time can improve reaction efficiency. If the distance between residues is greater than the cross-linker distance limit, then cross-linkers with longer backbones can be considered. Unpaired, covalent interaction of DSSO with a single Lys residue on a bait or target peptide indicates formation of a mono-link. Analysis of mono-links can provide information on surface accessibility of Lys residues.

DSSO in powder form is theoretically stable for years; however, if cross-links are not detected a new lot of DSSO can be used for cross-linking reactions.

Problem 4

No or low abundance of cross-links obtained by *in cellulo* cross-linking (steps 24–27).

Potential solution

If few or no cross-links are detected by *in cellulo* cross-linking compared to *in vitro* cross-linking, a different permeabilization technique, higher cross-linker concentration, or increased incubation

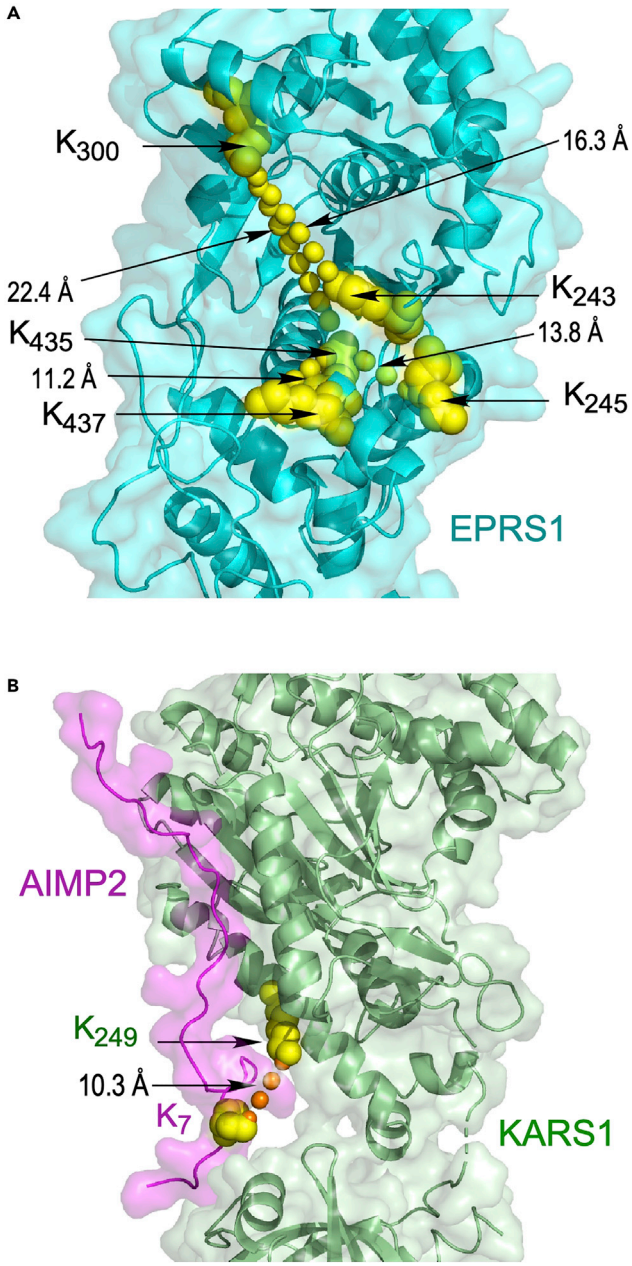

Figure 3. Validation of XL-MS-derived crosslinks for human MSC

(A) Intra-protein cross-link distances within structure of human EARS1 domain of EPRS1. EARS1 was modeled based on homology to the archaebacterium *Methanothermobacter thermautotrophicus* EARS1 (PDB ID: 3AII). Cross-linked Lys residues are shown as atom-level structures (yellow), and cross-links are indicated (line of yellow spheres).

(B) Inter-protein cross-link distances between AIMP2 and KARS1 in X-ray crystal structure (PDB ID: 6ILD). XL-MS-derived cross-linked Lys residues are highlighted (yellow spheres) and Inter-protein cross-links indicated (line of orange spheres). Figure reprinted with permission from Khan et al. (2020).

time can be adopted to increase cross-linking efficiency. Also, certify that the cross-linker is membrane permeable. To enrich low-abundance cross-linked peptides and separate them from unmodified peptides – a common problem associated with *in cellulo* cross-linking - other approaches can be used, e.g., biotinylated cross-linkers trapped on avidin beads (Kang et al., 2009; Tang and Bruce, 2010).

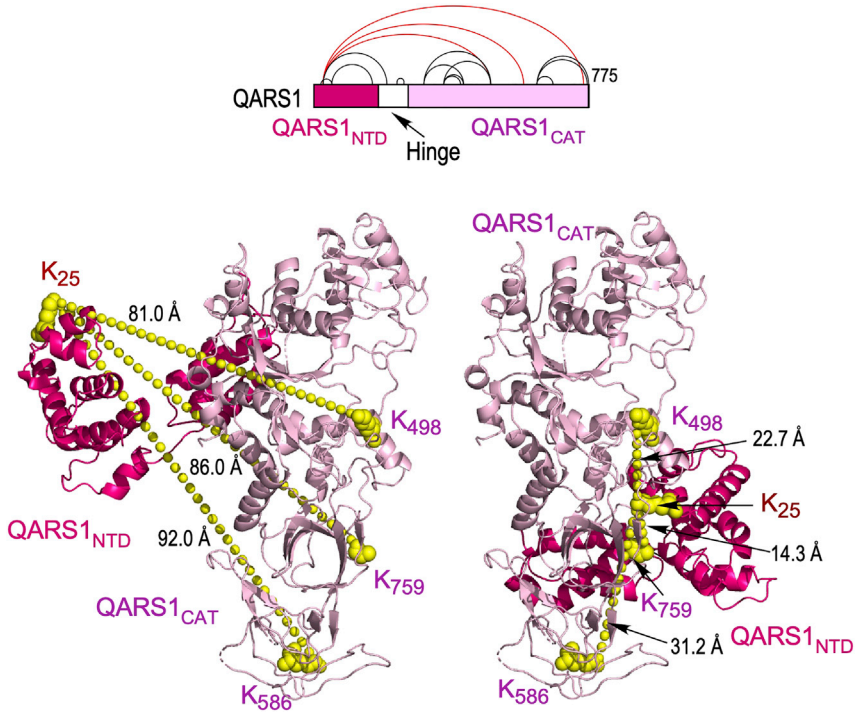

Figure 4. Adjustment of QARS1 structure by XL-MS-derived intra-links

(Top) XL-MS-derived intra-links in QARS1; cross-links between N-terminal domain (NTD) and catalytic domain (CAT) are highlighted (red). (Left) Crystal structure (PDB ID: 4YE6) of the NTD (red) and CAT (pink) of QARS1 showing 3 cross-links much greater than the ~27 Å limit (left). (Right) Amended model of QARS1 with XL-MS-derived cross-links within distance constraints of the cross-linker. Figure reprinted with permission from Khan et al. (2020).

Problem 5

Cross-links are not detected during mass spectrometry analysis (steps 35–46).

Potential solution

Presence of strong chromatogram peaks derived from protein complex of interest but no cross-links: Look for mono-links in bait or target protein as described in Problem 3. Absence of mono-links will indicate either no surface exposed Lys residues or failure of cross-linking reaction and will need further optimization.

Presence of mono-links will indicate absence of proximate intra- or inter-molecular Lys residues.

Absence of strong chromatogram peaks from protein complex of interest and no cross-links: This indicates insufficient enrichment of protein-complex components. More starting material or efficient pull-down conditions needs to be established under these scenarios.

Problem 6

Accommodation of all cross-links results in prohibited steric interference of domains during modeling (step 51).

Potential solution

The cross-link can be due to an alternative conformation of the holo-complex or to the presence of the target protein in a distinct, but related complex. If the former, an alternative conformation of one or more constituents, or a major alteration to the holo-model, can relieve the interference. If the latter, isolation, capture and analysis of the alternative complex might be necessary. Lastly, there

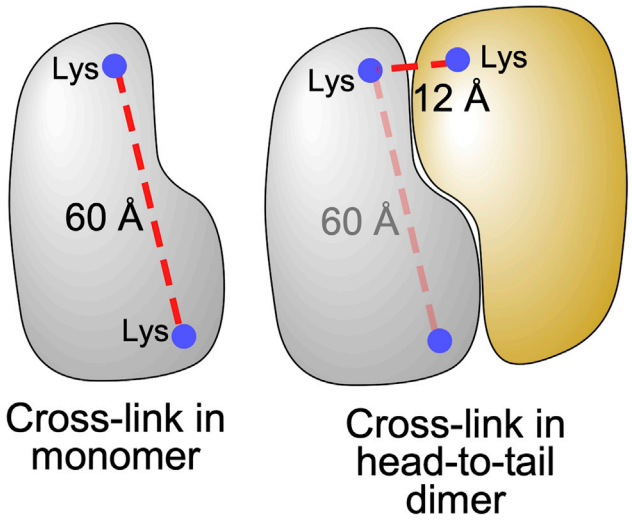

Figure 5. XL-MS-derived cross-links can provide evidence for homo-dimers
(Left) Cross-link (red dashed line) in monomeric structure exceeds distance constraint of the cross-linker. (Right) Cross-link in dimeric structure is within distance constraint of the cross-linker.

remains the possibility of a cross-link artifact; in this case a low-reliability cross-link, e.g., weakly detected by mass spectrometry, might need to be discarded.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Paul Fox (foxp@ccf.org).

Materials availability

This study did not generate specialized materials.

Data and code availability

This study did not generate datasets or code for any algorithms.

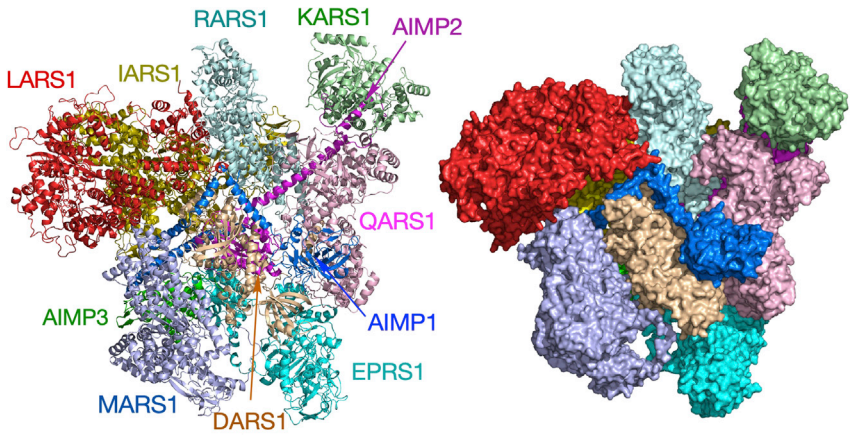

Figure 6. XL-MS-derived 3-D architecture of the human MSC
(Left) XL-MS-derived ribbon model of the human MSC. (Right) Space-fill model of the human MSC. Figure reprinted with permission from Khan et al. (2020).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101201.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.K. and P.F.; Methodology, K.K.; Investigation, K.K.; Analysis, K.K. and B.W.; Writing – O.D., K.K.; Writing - Review & Editing, K.K. and P.F.; Bioinformatic Analysis and Structural Modeling, C.B.-G. and V.G.; Supervision, Project Administration, and Funding Acquisition, P.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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