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Cross-linking/MS-analysis of Thr72-phosphorylated Rab8A and PPM1H (D288A) complex

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ABSTRACT

A subset of Rab proteins, including Rab8A, have been identified as substrates of the Leucine Rich Repeat Kinase 2 (LRRK2) and the Protein Phosphatase PPM1H (Steger et al. 2017; Berndsen et al. 2019). As the crystal structure of the complex of PPM1H with Rab8A pT72 is unavailable, we employed a cross-linking approach (Iacobucci et al. 2018) to perform a study to identify the topology of this phosphatase-substrate binding. A 'substrate-trapping' mutant (D288A) of PPM1H was identified (Berndsen et al. 2019), and used in this study to form stable complex of PPM1H with Rab8A pT72. We then used Disuccinimidyl Dibutyric Urea (DSBU) to cross-link the complex of PPM1H (D288A) 'substrate-trapping' mutant and phospho-Rab8A. DSBU is a mass spectrometry cleavable amine reactive crosslinker with a 12.5 Å spacer arm that is widely used to identify and map sites of protein-protein interactions (Pan et al. 2018). This reagent cross-links Lys residues to acidic and hydroxyl amino acids located within 32 Å (Götze et al. 2019). Crosslinked samples were excised from a Coomassie-stained poly acrylamide gel and digested with 3 conditions (trypsin, trypsin/Asp-N and trypsin/Glu-C). In addition, SCX cartridge purification was applied in one of the tryptic digested samples to further enrich the cross-linked peptides. PPM1H and pRab8a cross-linked peptides were identified using meroX software (Götze et al. 2012). Potential crosslinked peptides with score higher than 50 as well as false discovery rate (FDR) less than 5% were manually inspected to confirm only a single crosslinked site was proposed from each peptide (Iacobucci et al. 2018).

ATTACHMENTS

duvxbgrdf.pdf

DOI

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PROTOCOL CITATION

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MATERIALS TEXT

Consumables:

- Recombinant Protein - PPM1H (1 - 514) D288A **MRC PPU Reagents and Services Catalog #DU68087** in 40 mM HEPES pH 7.5 or PBS. Note it is critical that no Tris or other amine containing buffers are present in the protein prep, as this could interfere with the assay. A buffer exchange into [M]**40 Milimolar (mM)** HEPES pH**7.5**, [M]**150 Milimolar (mM)** NaCl, [M]**2 Milimolar (mM)** MgCl2 buffer is needed prior to the cross-linking (described as the first step of this protocol). Wild-type PPM1H phosphatase (DU62835) can be used as a control.
- Recombinant stoichiometrically Thr72 phosphorylated Rab8A (described in protocols.io dx.doi.org/10.17504/protocols.io.butinwke or purchased https://mrcppureagents.dundee.ac.uk/ (DU68198). Note it is critical that no Tris or other amine containing buffers are present in the protein prep, as this could interfere with the assay. A buffer exchange into [M]**40 Milimolar (mM)** HEPES pH**7.5**, [M]**150 Milimolar (mM)** NaCl, [M]**2 Milimolar (mM)** MgCl2 buffer is needed prior to the cross-linking (described as the first step of this protocol). Non-phosphorylated Rab8A can be used as a control.
- Tris(hydroxymethyl)aminomethane (TRIS Trometamol) 99.8-100.5% AnalaR® NORMAPUR® analytical reagent **VWR Chemicals Catalog #103157P**
- HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid) ≥99.5% for biochemistry **VWR Chemicals Catalog #441487M**
- Magnesium chloride hexahydrate **Sigma Aldrich Catalog #M2670**
- Sodium chloride 99.5-100.5% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR Chemicals Catalog #27810.364**
- DSBU (Disuccinimidyl Dibutyric Urea) **Thermo Fisher Catalog #A35459**
- Dimethyl sulfoxide anhydrous, >= 99.9 % | 67-68-5 | Sigma-Aldrich?

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Dimethyl sulfoxide **Sigma**

Aldrich Catalog #D8779 or equivalent

Seq Grade Modified Trypsin, 100ug (5 x

- 20ug) **Promega Catalog #V5111**

Asp-N, Sequencing Grade,

- 2ug **Promega Catalog #V1621**

Endoproteinase Glu-C Sequencing Grade **Merck**

- **Millipore Catalog #11420399001**
- ThermoFisher NP0321BOX 4-12% NuPAGE Bis-Tris 1mm 10-well gel

Colloidal Blue Staining Kit **Thermo**

- **Fisher Catalog #LC6025**

NuPAGE™ LDS Sample Buffer (4X) **Invitrogen - Thermo**

- **Fisher Catalog #NP0008**

Iodoacetamide **Sigma**

- **Aldrich Catalog #I1149-5G**
- Acetonitrile **Merck Catalog #1.00030.2500**

Ammonium acetate **Sigma**

- **Aldrich Catalog #09688**

Formic acid 98-

- 100% **Merck Catalog #1.00263.1000**
- **Methanol (MeOH; LC-MS grade; MERCK; cat. no. 1.06035.1000)**

Acetic acid glacial ≥99.7% ACS **VWR**

- **Chemicals Catalog #36289.K3**
- **Phosphoric acid (H3PO4; 85% (v/v) solution; Sigma-Aldrich; cat. no. 345245)**

Trifluoroacetic acid for HPLC > 99.0% **Sigma-**

- **aldrich Catalog #302031-100ML**

Ammonium bicarbonate **Sigma**

- **Aldrich Catalog #09830-500G**

Solutions (all made fresh):

- [M]**40 Milimolar (mM)** HEPES pH**7.5**, [M]**150 Milimolar (mM)** NaCl, [M]**2 Milimolar (mM)** MgCl2 in milli-Q water
- [M]**1 Molarity (M)** Tris pH**8.8** in milli-Q water
- [M]**100 Milimolar (mM)** ABC (Ammonium bicarbonate) in milli-Q water
- [M]**25 Milimolar (mM)** ABC (Ammonium bicarbonate) in milli-Q water
- [M]**10 Milimolar (mM)** DTT in milli-Q water
- [M]**55 Milimolar (mM)** IAA in 100 mM ABC
- [M]**50 Milimolar (mM)** ABC in 50% (v/v) acetonitrile (prepared my mixing equal volumes of [M]**100 Milimolar (mM)** ABC and ACN)
- 5% (v/v) TFA in milli-Q water
- 1.67% (v/v) TFA in acetonitrile (prepared by mixing 2 volumes of ACN with 1 volume of 5% (v/v) TFA solution)

Equipment:

- **2 µl**, **20 µl**, **200 µl** and **1000 µl** Gilson pipettes

Protein LoBind

- **1.5 mL** tubes **Eppendorf Catalog #022431081**
- Diacon **0.5 mL** 10 kDa MWCO dialysis tube (MD6-71, Molecular Dimensions)
- Eppendorf Thermomixer
- Magnetic stirrer with stir bars
- Nanodrop 1000 (Thermo Fisher Scientific)
- SpeedVac (SPD140DDA; Thermo Fisher Scientific)
- MCX Cartridge (OASIS PRiME MCX 1 cc cartridge; Waters Corporation)

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- HPLC analytical column (PepMapTM RSLC C18, 75 µm x 50 cm, 2 µm, 100 Å; Thermo Fisher Scientific)
- HPLC trap column (Acclaim PepMapTM 100, C18, 100 µm x 2 cm, 5 µm, 100 Å; Thermo Fisher Scientific)
- HPLC system (Ultrimate 3000 RSLC nano-HPLC; Thermo Fisher Scientific)
- Mass spectrometer (Orbitrap ExplorisTM 480; Thermo Fisher Scientific)
- Water bath (Bandelin Electronic)

Software:

- MeroX software 2.0

Protein buffer exchange 4h

1

As the cross-linking reaction can be inhibited by amine containing buffers, a buffer exchange step for both, PPM1H and pRab8A protein solutions is needed prior to the crosslinking. Proteins should be dialysed into 40 mM HEPES pH 7.5, 150 mM NaCl, 2 mM MgCl2 buffer.

Prepare a beaker containing **500 mL** of **40 Milimolar (mM)** HEPES **7.5**, **150 Milimolar (mM)** NaCl, **2 Milimolar (mM)** MgCl2 buffer and a magnetic stir bar. Set the beaker on a magnetic stirrer with gentle stirring, at **Room temperature** .

2 2h

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Pipette up to **0.5 mL** of each protein into separate Diacon dialysis tubes. Put the tubes in a floating rack, and place the rack on the surface of the buffer in the beaker. Incubate for **02:00:00** at **Room temperature** .

3 2h

Transfer the floating rack with the dialysis tubes into a fresh beaker containing **500 mL** of **40 Milimolar (mM)** HEPES **pH 7.5** , **150 Milimolar (mM)** NaCl, **2 Milimolar (mM)** MgCl2 buffer and a magnetic stir bar. Set the beaker on a magnetic stirrer with gentle stirring, at **Room temperature** . Incubate **Overnight** at **Room temperature** .

Preparation of the cross-linked pRab8A/PPM1H complex samples 1h 25m

4 Prepare a solution of stoichiometrically Thr72 phosphorylated Rab8A at a concentration **35 Micromolar (µM)** (~ **0.7 mg/ml** , migrates at 20 kDa) and PPM1H at **28 Micromolar (µM)** (~ **1.4 mg/ml** , migrates at 50 kDa) in total volume of **15.5 µl** using **40 Milimolar (mM)** HEPES **pH 7.5** , **150 Milimolar (mM)** NaCl, **2 Milimolar (mM)** MgCl2 as a dilution buffer, to achieve 1.25-fold molar excess of Rab8A to PPM1H.

The mixed volumes will depend on protein concentrations obtained after the buffer exchange step.

5 1h

Mix the protein solution gently and incubate at **30 °C** for **01:00:00** .

6 10m

After this incubation incubate at **Room temperature** for **00:10:00** .

7 5m

During this time prepare a fresh **300 Milimolar (mM)** stock solution of the cross-linker by dissolving **1 mg** of DSBU in **15.5 µl** of anhydrous DMSO, vortex briefly, keep **On ice** , use within **00:05:00** .

8

Add **0.5 µl** of the cross-linker solution into **15.5 µl** of the protein solution, mix gently.

This will result in final concentration of **9.375 Milimolar (mM)** of DSBU (250-fold molar excess to pRab8A, 335-fold molar excess to PPM1H) in **16 µl** of reaction mix.

9 10m

Incubate the cross-linking reaction for **00:10:00** at **Room temperature** . Add **2 µl** of **1 Molarity (M)** Tris **pH 8.8** to quench the reaction, mix gently.

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10

Add **7 µl** of 4 x NuPAGE™ LDS Sample Buffer (containing lithium dodecyl sulfate at pH **8.5** with SERVA Blue G250 and phenol red), vortex. Immediately resolve the samples using SDS-PAGE.

Do not add reducing agent such a 2-mercaptoethanol or heat the samples before electrophoresis as this could impact crosslinking reactions.

SDS-PAGE and in-gel digestion 20m

11 20m

Resolve the samples on a 4-12% gradient NuPAGE Bis-Tris gel. The final reaction volume is **25 µl** . Load **6 µl** aliquots of each sample into 4 lanes (one for each protease condition: Sample A – trypsin, Sample B – trypsin+SCX, Sample C – trypsin+AspN, Sample D – trypsin+GluC). Run the gel at 70 V for **00:20:00** , and then at 120 V until the dye front leaves the gel.

12 Fix and stain the gel using Invitrogen™ Colloidal Blue Staining Kit

12.1

Prepare the fixing solution (10% (v/v) glacial acetic acid in 50% (v/v) methanol) by mixing **5 mL** of acetic acid with **25 mL** of methanol and **20 mL** of milliQ water.

12.2 10m

Incubate the gel in the fixing solution for **00:10:00** at **Room temperature** with gentle shaking. Discard the fixing solution.

12.3 10m

Prepare the staining solution (20% (v/v) methanol, 20% (v/v) Stainer A, 5% (v/v) Stainer B). Mix **10 mL** of Stainer A with **10 mL** of methanol and **22.5 mL** of milli-Q water. Do not add Stainer B at this stage. Incubate the gel in the solution for **00:10:00** at **Room temperature** with gentle shaking.

12.4 6h

Add **2.5 mL** of Stainer B directly to the solution containing the gel. Incubate the gel in the staining solution for **02:00:00** – **04:00:00** at **Room temperature** with gentle shaking.

12.5 8h

Rinse the gel using milli-Q water at least 3 times within at least **04:00:00** or **Overnight** .

12.6 Image the gel.

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Note: Band-shifts corresponding to a PPM1H dimer size (100 kDa), and a tetramer of PPM1H and pRab8A (140 kDa) are an indication of a successful cross-linking (Figure 1). Decide which crosslinked species need to be excised. We excised the pRab8A-PPM1H crosslinked species and the PPM1H dimer crosslinked species highlighted on Figure 1.

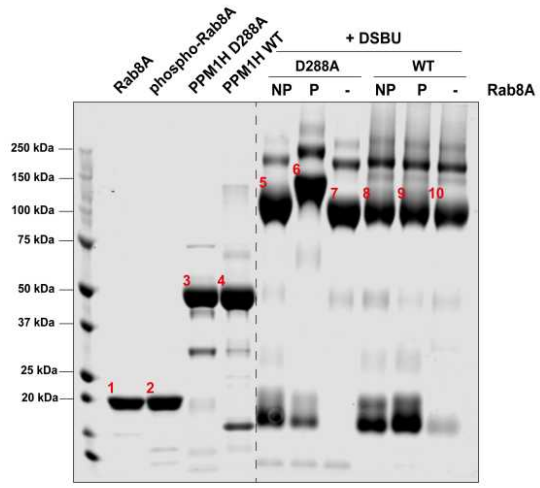

Figure 1. Coomassie-stained SDS-PAGE gel showing the result of cross-linking of PPM1H D288A with phospho-Rab8A. Protein bands on the gel – 1: non-phopsho-Rab8A; 2: phospho-Rab8A; 3: PPM1H(D288A); 4: PPM1H(WT); 5: Cross-linked dimer of PPM1H(D288A); 6: Cross-linked heterotetramer of PPM1H(D288A) and phospho-Rab8A; 7: Cross-linked dimer of PPM1H(D288A); 8-10: Cross-linked dimer of PPM1H(WT)

13 Prepare low-binding **1.5 mL** Eppendorf tubes – one for each condition (A, B, C, D).

14 Using a fresh, sterile scalpel, excise each band of interest, cut into **1 mm** cubes, and place the gel pieces in a.

15 10m

Add **500 µl** of acetonitrile and incubate at **Room temperature** for **00:10:00** to dehydrate the gel pieces.

16 Discard the supernatant.

17

Add **50 µl** of freshly prepared **10 Milimolar (mM)** DTT solution and ensure gel pieces are covered. If necessary, add an additional **50 µl** of **10 Milimolar (mM)** DTT to cover gel pieces.

18 30m

Incubate in a Thermomixer at **56 °C** for **00:30:00** with mixing (**1200 rpm**).

19

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Add **500 µl** of acetonitrile.

20 10m

Incubate at **Room temperature** for **00:10:00** without shaking to dehydrate the gel pieces.

21 Discard the supernatant.

22 Prepare a fresh solution of **55 Milimolar (mM)** Iodoacetamide (IAA) in **100 Milimolar (mM)** ABC.

Please note that iodoacetamide is toxic and should be carefully weighed out in a fume hood (for details refer to the safety data sheet).

23

Add **50 µl** - **100 µl** of freshly prepared **55 Milimolar (mM)** iodoacetic acid solution to ensure that gel pieces are covered.

24 Cover reaction mixture from light by covering the tubes with aluminium foil.

25 20m

Incubate in a Thermomixer at **Room temperature** for **00:20:00** with mixing (**1200 rpm**).

26

After this incubation add **500 µl** of acetonitrile.

27 10m

Incubate at **Room temperature** for **00:10:00** to dehydrate the gel pieces without shaking.

28 Discard the supernatant.

Please note that the solution contains toxic iodoacetamide and should be disposed of in an appropriate manner. For details refer to the safety data sheet.

29

Add **100 µl** of **50 Milimolar (mM)** ABC in 50% (v/v) acetonitrile.

30m

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30 Incubate at **Room temperature** for **00:30:00** without shaking.

31 Add **500 µl** of acetonitrile. Do not discard the supernatant from the previous step.

32 10m

Incubate at **Room temperature** for **00:10:00** to dehydrate the gel pieces.

33 Discard the supernatant.

34 Prepare fresh **80 µl** solution of **[M]50 ng/µl** Trypsin in milli-Q water keep **On ice** .

35 Prepare fresh **20 µl** solution **[M]20 ng/µl** , AspN in milli-Q water keep **On ice** .

36 Prepare fresh **20 µl** solution **[M]50 ng/µl** , GluC in milli-Q water keep **On ice** .

37 Use 4 different protease digestion conditions: Sample A – trypsin, Sample B – trypsin+SCX enrichment, Sample C – trypsin+AspN, Sample D – trypsin+GluC.

38 For A add **20 µl** of Trypsin solution to the dehydrated gel pieces. If the gel pieces are not covered completely, add **[M]25 Milimolar (mM)** ABC solution to cover them.

39 For B add **20 µl** of Trypsin solution to the dehydrated gel pieces. If the gel pieces are not covered completely, add **[M]25 Milimolar (mM)** ABC solution to cover them.

40 For C add **20 µl** of Trypsin solution to the dehydrated gel pieces, followed by **20 µl** of AspN solution. If the gel pieces are not covered completely, add **[M]25 Milimolar (mM)** ABC solution to cover them.

41 For D add **20 µl** of Trypsin solution to the dehydrated gel pieces, followed by **20 µl** of GluC solution. If the gel pieces are not covered completely, add **[M]25 Milimolar (mM)** ABC solution to cover them.

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42 14m

Incubate all 4 samples **Overnight** at **30 °C** with mixing (**1200 rpm**).

43

Prepare fresh peptide elution solution (1.67% (v/v) TFA in acetonitrile) by mixing 2 volumes of ACN with 1 volume of 5% (v/v) TFA solution.

44

Add **100 µl** peptide elution solution to each of the digestion tubes.

45 15m

Incubate at **37 °C** for **00:15:00** with mixing (**1200 rpm**).

46 Transfer the supernatant that contains the digested protein into a fresh **1.5 mL** low-binding Eppendorf tube. Avoid collecting any gel pieces.

47 In order to extract the remaining peptides present in the gel pieces, repeat the elution step by adding another **100 µl** into the gel pieces.

48 15m

Incubate at **37 °C** for **00:15:00** .

49 Collect the supernatant and combine with the previously collected supernatant in step 46.

50 Freeze the samples on dry ice.

51 Vacuum dry using SpeedVac.

MCX SPE cartridge for cross-linked peptide enrichment 30m

52 30m

MCX cartridge contains mixed-mode sorbent (reversed-phase and cation exchange) which provides higher selectivity and sensitivity for cross-linked peptides enrichment than traditional SCX cartridge (Schmidt and Sinz 2017). Herein, we applied a similar protocol as previous study to enrich one of our tryptic digested samples (Iacobucci et al. 2018). Take tube labelled B from Freeze dried sample from step 51 and add **1 mL** aqueous solution containing 4% (v/v) H_3PO_4.

Place the tube in a water bath sonicator at **Room temperature** for **00:30:00** .

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53

Wash the MCX cartridge with **2 mL** methanol (no syringe or manifold was applied; according to the nature protocol (Iacobucci et al. 2018), slow sample loading can enhance the interaction between the cross-linked peptides and cartridge). Wash the MCX cartridge with 2 aqueous solution containing 4% (v/v) H_3PO_4.

54

Load sample from step 12 using a **1000 µl** pipette onto the washed MCX cartridge.

55

Wash the MCX cartridge with **500 µl** aqueous solution containing 4% (v/v) H_3PO_4.

56

Wash the MCX cartridge with **500 µl** of 10% (v/v) MeOH solution with 0.1% (v/v) FA.

57

Elute low-charged peptides by washing the MCX cartridge with **500 µl** solution composed of **500 Milimolar (mM)** NH_4OAc in 40% (v/v) MeOH solution with 0.1% (v/v) FA.

58

Elute high-charged peptides by washing the MCX cartridge with **700 µl** solution composed of **2000 Milimolar (mM)** NH_4OAc in 80% (v/v) MeOH solution with 0.1% (v/v) FA.

59 Freeze the samples on dry ice.

60 Vacuum dry using SpeedVac

LC MS/MS experiment

61

Liquid chromatography tandem mass spectrometry (LC MS/MS) experiment was performed on an Ultrimate 3000 RSLC nano-HPLC system coupled to an Orbitrap ExplorisTM 480 mass spectrometer.

Re-suspend all vacuum dried samples in **30 µl** solution containing 3% (v/v) acetonitrile and 0.1% (v/v) FA.

62 Place tube in a water bath sonicator at **Room temperature** for **00:30:00** . 30m

63 Load **3 µl** – **14 µl** solution from each sample onto the nano-HPLC system individually. Trap the peptides by a 2h

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precolumn (Acclaim PepMapTM 100, C18, 100 µm x 2 cm, 5 µm, 100 Å) using aqueous solution containing 0.1% (v/v) TFA. Seperate the peptides by an analytical column (PepMapTM RSLC C18, 75 µm x 50 cm, 2 µm, 100 Å) at **45 °C** using a linear gradient of 1 to 35% solvent B (solution containing 80% (v/v) acetonitrile and 0.1% (v/v) FA) for **01:30:00** , 35 to 85% (v/v) solvent B for **00:05:00** , 85% (v/v) solvent B for **00:10:00** , 85 to 1% (v/v) solvent B for **00:01:00** , and 1% (v/v) solvent B for **00:14:00** . Set the flow rate at **300 nL/min** for all experiments.

64 Acquire the data with data-dependent MS/MS mode. For each MS scan, set the scan range between 375 and 1500 m/z with the resolution at 120,000 and use 300% automatic gain control (AGC).

The maximum injection time for each MS scan was 100 ms. The 10 highest abundant peptides with charge state between 2 and 8 as well as intensity threshold higher than 1.0e+4 were then isolated with a 1.2 Da isolation window sequentially. Stepped HCD with normalized collision energy of 27, 30, and 33% was applied to fragment the isolated peptides. For each MS/MS scan, the resolution was set at 15,000 with a normalized AGC at 200%. The maximum injection time was set at 250 ms.Dynamic exclusion with 60 s duration and 2 ppm window was enabled for the experiment.

Cross-linked peptide data analysis

65 Convert the .RAW files obtained from the LC MS/MS experiments into .mgf files using RawConverter software (He et al. 2015).

66 Submit the .mgf files to search using MeroX software against PPM1H and Rab-8A protein sequences to identify potential cross-linked peptides (He et al. 2015).

Digestive enzyme, trypsin, trypsin and AspN, or trypsin and GluC, were selected according to the experimental setup. 3 maximum missed cleavages with peptide length ranged from 3 to 50 were applied. Carbamidomethylation at Cysteine residue was set as fixed modification; while oxidation at methionine residue and deamidation at asparagine residue were included in variable modification. DSBU cross-linker was selected with specificity cross-linked sites at Lysine, Serine, Threonine, and Tyrosine residues. 10 ppm and 20 ppm were used to filter the mass error in precursor ion (MS1) and fragment ion (MS2) scans. Only ions with signal-to-noise ratio high than 2 were used for database search. RISEUP searching mode was applied, minimum 2 fragments per peptide and 5% false discovery rate (FDR) were required for a cross-linked peptide identification.

67 Verify the potential cross-linked peptides with score higher than 50 to guarantee the cleavage information obtained from the MS experiment (Figure 2 and 3).

Citation: Pawel Lis, Pui Yiu Lam, Axel Knebel , Kerryn Berndsen, Dario R Alessi (06/29/2021). Cross-linking/MS-analysis of Thr72-phosphorylated Rab8A and PPM1H (D288A) complex. https://dx.doi.org/10.17504/protocols.io.bv2en8be

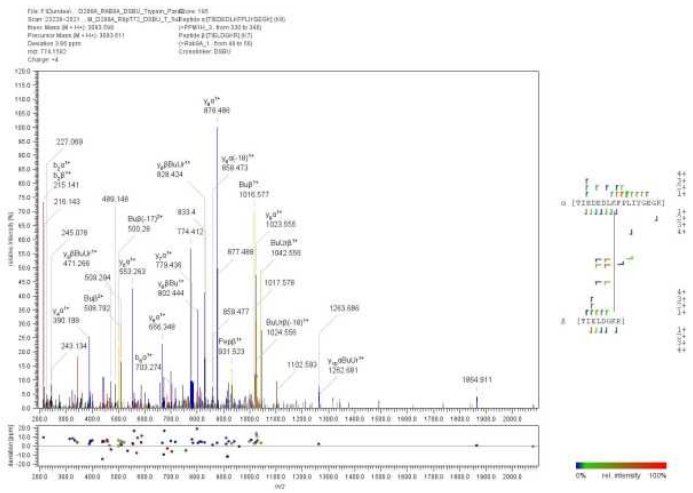

Figure 2. High confident crosslinked PPM1H and pRab8A peptides were identified in the tryptic digested sample with calculated score (by meroX software) equal to 181 and FDR < 0.05. Fragments obtained from the MS spectrum proposed the interaction was located at K8 (PPM1H peptide) and K7 residue (pRab8A peptide). We accepted this crosslinked peptide and included this information in our further analysis since a single interaction site was proposed from each peptide.

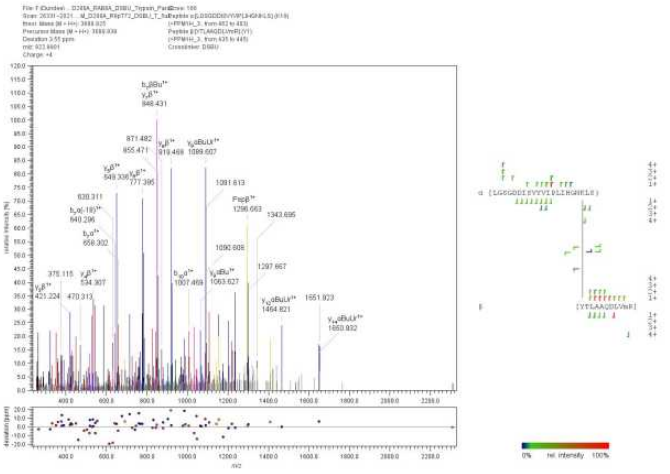

Figure 3. Ambiguous crosslinked PPM1H and pRab8A peptides were found in the tryptic digested sample with the calculated score (by meroX software) equal to 151 and FDR < 0.05. Fragments obtained from the MS spectrum proposed the interaction was located at K19 residue from PPM1H peptide; the fragmentation information for pRab8A peptide, however, could not differentiate the interaction between Y1 and T2 residues. In this situation, we rejected the peptide and excluded this crosslink information for the further analysis.

Citation: Pawel Lis, Pui Yiu Lam, Axel Knebel , Kerryn Berndsen, Dario R Alessi (06/29/2021). Cross-linking/MS-analysis of Thr72-phosphorylated Rab8A and PPM1H (D288A) complex. https://dx.doi.org/10.17504/protocols.io.bv2en8be