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Identification of DNA damage checkpoint-dependent protein interactions in *Saccharomyces cerevisiae* using quantitative mass spectrometry

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Summary

The DNA damage checkpoint (DDC) is an evolutionarily conserved signaling pathway that is crucial to maintain genomic integrity. In response to DNA damage, DDC kinases are rapidly activated and phosphorylate an elaborate network of substrates involved in multiple cellular processes. An important role of the DDC response is to assemble protein complexes. However, for most of the DDC substrates, how the DDC-dependent phosphorylation modulates their network of interactions remains to be established. Here, we present a protocol for the identification of DDC-dependent protein-protein interactions based on Stable Isotope Labeling of Amino acids in Cell culture (SILAC) followed by affinity-tagged protein purification and quantitative mass spectrometry analysis. Based on a model study using *Saccharomyces cerevisiae*, we provide a method that can be generally applied to study the role of kinases in mediating protein-protein interactions.

Keywords

Protein-protein interaction; DNA damage checkpoint; *Saccharomyces cerevisiae*; SILAC; Affinity tagged protein purification; Quantitative mass spectrometry

1. Introduction

In response to DNA damage, eukaryotic cells trigger a highly conserved signaling pathway known as the DNA damage checkpoint (DDC). The DDC is critical to prevent the accumulation of genomic instability and cancer [1–3]. DDC signaling is initiated by the upstream PI3K-like sensor kinases Mec1 and Tel1 (orthologs of human ATR and ATM, respectively) [4]. Once activated, these kinases phosphorylate numerous proteins to coordinate a wide range of cellular processes including DNA repair, cell cycle progression, transcription regulation and DNA replication among others [5–8].

A prevalent role for phosphorylation in the DDC response is believed to be the assembly of protein complexes [9–11]. Consistent with this notion, many proteins that participate in the DDC response contain phosphopeptide binding domains, such as BRCT (BRCA1 C-

Terminus) or FHA (Forkhead-Associated), that recognize sites phosphorylated by DDC kinases [12,13]. Recently, ATR/ATM or Mec1/Tel1-dependent phosphorylation sites have been identified in hundreds of proteins [14–16], but in the vast majority of these substrates the role of phosphorylation is not known. In most cases, establishing whether these phosphorylation events are modulating the network of interactions of the substrates will be important to understand how the DDC coordinates the DNA damage response.

Mass spectrometry analysis of protein pull-down assays has been widely used for the identification of protein-protein interactions [17]. However, due to the high sensitivity of mass spectrometry, any pull-down assay results in identification of a large number of non-specific background proteins. Recently, our laboratory developed a two-step protocol based on quantitative mass spectrometry analysis to identify protein-protein interactions that are mediated by the DDC (*see Fig. 1*). In the first step we combine SILAC [18] and protein affinity tag purification to compare the relative abundance of co-purified proteins between a tagged or untagged version of any protein of interest. This step allows the discrimination between background and specific interactions with the protein of interest (*see Fig. 1A*). In the second step, we compare the relative abundance of co-purified proteins between a wild type and a checkpoint kinase null mutant strain to reveal kinase-dependent interactions (*see Fig. 1B*).

For the general purpose of this protocol, here we use the Mec1-dependent interactions mediated by the BRCT domain-containing protein Rtt107 as an example [10]. However, with minor modifications, this protocol can be adapted to provide a reliable method for the identification of protein-protein interactions in budding yeast.

2. Materials

2.1. Stable Isotope Labeling of Amino acids in Cell culture (SILAC)

1. Strains MBS164 (*MATa, ura3-52, leu2⁻1, trp1⁻63, his3⁻200, lys2⁻Bgl, hom3-10, ade2⁻1, ade8, arg4⁻, sml1::TRIP1, bar1::HIS3*); MBS266 (*MATa, ura3-52, leu2⁻1, trp1⁻63, his3⁻200, lys2⁻Bgl, hom3-10, ade2⁻1, ade8, arg4⁻, sml1::TRIP1, bar1::HIS3, Rtt107-6xhis-3xHA::KanMX6*) and MBS402 (*MATa, ura3-52, leu2⁻1, trp1⁻63, his3⁻200, lys2⁻Bgl, hom3-10, ade2⁻1, ade8, arg4⁻, sml1::TRIP1, bar1::HIS3, Rtt107-6xhis-3xHA::KanMX6, mec1::URA3*) (*see Note 1*).
2. SD –Arg –Lys media for SILAC: 0.67 g of CSM-Arg-His-Lys (Sunrise Science) + 6.7 g of YNB + 80 mg of L-Proline + 40 mg of L- Histidine + 960 mL of distilled water. After autoclave, add 40 mL of a 50 % dextrose solution to a final concentration of 2% (*see Note 2*).

¹Strains MBS164, MBS266 and MBS402 are SILAC auxotrophic strains for Lysine and Arginine and are available upon request. These strains carry a deletion of the ribonucleotide reductase inhibitor *SML1* which suppresses the lethality of *mec1⁻* allele [22].

²Proline is added to prevent the metabolic conversion of heavy arginine to heavy proline.

3. 1000 × “Light” Arg/Lys stock solution: 30 mg/mL of L-Arginine (0.2 M) + 20 mg/mL of L-Lysine (0.115 M). Dissolve amino acids in distilled water and filtrate using a 0.22 µm disposable syringe filter.
4. 1000 × “Heavy” Arg/Lys stock solution: 38 mg/mL of L-Arginine ¹³C₆, ¹⁵N₄ (0.2 M) (Sigma Aldrich) + 25 mg/mL of L-Lysine ¹³C₆, ¹⁵N₂ (0.115 M) (Sigma Aldrich). Dissolve amino acids in distilled water and filtrate using a 0.22 µm disposable syringe filter.
5. To prepare 1 L of SD “Light” or SD “Heavy” add 1 mL of 1000 × “Light” Arg/Lys or 1000 × “Heavy” Arg/Lys stock solution to 1 L of SD –Arg –Lys.

2.2. Methyl Methanesulfonate (MMS)-Induced Replication Stress and Cells Harvesting

1. Methyl methanesulfonate (Sigma Aldrich).
2. TE buffer: 10 mM Tris-HCl pH 8.0, 5 mM EDTA.

2.3. Cell Lysis

1. Lysis buffer: 50 mM Tris-HCl pH 7.5, 0.2 % Tergitol, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 × complete EDTA-free protease inhibitor cocktail (Roche) and 1 × phosphatase inhibitor (*see* Note 3).
2. 100 × phosphatase inhibitor: 100 mM Na₃VO₄, 500mM NaF and 1 M β-glycerophosphate.
3. Glass beads, 0.05 mm dia. (BioSpec).
4. Protein assay dye reagent (Bio-Rad).
5. Cell disruptor Genie™ (Scientific Industries).

2.4. Immunoprecipitation of 3xHA-tagged Rtt107

1. EZview™ red anti-HA affinity gel (Sigma Aldrich).
2. Elution Buffer: 100 mM Tris-HCl pH 8.0, 1% sodium dodecyl sulfate, 10 mM dithiothreitol (DTT) (*see* Note 4).
3. Micro Bio-Spin® chromatography columns (Bio-Rad).

2.5. Protein Precipitation and Tryptic Digestion

1. Alkylation solution: 1 M Tris-HCl pH 8.0, 0.5 M iodoacetamide (*see* Note 5).
2. Precipitation solution: 50 % acetone, 49.9 % ethanol, 0.1 % biochemistry grade acetic acid.
3. Water bath sonicator (Branson 2510).
4. Urea/Tris solution: 1 M Tris pH 8.0, 8 M urea (*see* Note 5).

³Add PMSF, EDTA-free protease inhibitor cocktail and phosphatase inhibitor to cell lysis buffer right before proceeding to cell lysis.

⁴Add DTT to protein elution buffer right before proceeding to protein elution.

⁵Prepare a fresh alkylation solution for every experiment.

5. NaCl₂/Tris solution: 50 mM Tris-HCl pH 8.0, 150 mM NaCl₂.
6. Gold Trypsin® (Promega) 1 µg/µl in 0.3 % biochemistry grade acetic acid (*see* Note 6).

2.6. Protein Sample Clean-Up

1. 10% LC-MS grade formic acid (*see* Note 7).
2. 10% trifluoroacetic acid (*see* Note 7)
3. Sep-Pak® C₁₈ cartridges Vac 1 cc, 50 mg (Waters).
4. C₁₈ buffer A: 0.1 % trifluoroacetic acid (*see* Note 7).
5. C₁₈ buffer B: 0.1 % biochemistry grade acetic acid (*see* Note 7).
6. C₁₈ buffer C: 80 % HPLC grade acetonitrile, 0.1 % biochemistry grade acetic acid (*see* Note 7).
7. Polyspring®silanized conical base insert vial, 300 µl (National Scientific).

2.7. Hydrophilic Interaction Liquid Chromatography (HILIC)

1. This protocol assumes access to high performance liquid chromatography (HPLC) equipment including an elution gradient programmer with data processing software, UV absorbance detector, and automated fraction collector.
2. 99.9% HPLC grade acetonitrile (*see* Note 7)
3. Column: 2.0 × 150 mm TSK gel Amide-80 5 µm particle (Tosoh Bioscience).
4. Buffer D: 90 % HPLC grade acetonitrile (*see* Note 7).
5. Buffer E: 80 % HPLC grade acetonitrile with 0.005 % trifluoroacetic acid (*see* Note 7).
6. Buffer F: 0.025 % trifluoroacetic acid (*see* Note 7).

2.8. Reverse Phase Liquid Chromatography-Tandem Mass Spectrometry

1. The protocol assumes access to an on-line nano LC system interfaced to a mass spectrometer capable of performing tandem MS/MS.
2. Reverse phase analytical column: 125 µm ID × 20 cm (Polymicro Technologies) in-house packed with 3 µm C₁₈ resin (Michron Bioresources) (*see* Note 8)
3. Buffer I: 0.1 % LC-MS grade formic acid (*see* Note 7).
4. Buffer II: 0.1 % LC-MS grade formic acid with 80 % HPLC grade acetonitrile (*see* Note 7).

⁶After the trypsin is diluted in 0.3% acetic acid, keep small aliquots frozen at -80 °C.

⁷To avoid sample contamination with polymers always use a glass syringe/glass pipette/glass graduated cylinder to dilute strong acids and high concentrated organic solvents.

⁸The analytical column was generated by pulling capillary to 5 µm-ID tip. Reverse-phase particles were packed directly into the pulled column at 1000 psi until 20 cm long. The column was further packed, washed and equilibrated at 1000 psi. in buffer II followed by buffer I.

2.9. Database Searching and Protein Quantification

1. For database search: SEQUEST software on a SORCERER system (Sage-N Research).
2. For quantification of relative protein abundances: XPRESS software.

3. Methods

3.1. Stable Isotope Labeling of Amino acids in Cell culture (SILAC)

The basic principle of SILAC for quantitative proteomics analysis consists on growing two cultures of cells, one in a medium complemented with normal (“light”) amino acids and the other in a medium complemented with stable-isotope labeled (“heavy”) amino acids. The incorporation of “heavy” amino acids allows each peptide to be detected as a pair in the mass spectra, with a known mass shift compared with the peptide labeled with the “light” version. In this case, because the “light” and “heavy” amino acids are chemically identical, except for their mass difference, the ratio of peak intensities in the mass spectrometer directly yields the ratio of protein abundance between the two cultures.

1. For step 1 of the analysis, inoculate 100 mL of SD “light” and SD “heavy” media with fresh colonies from either strains MBS164 and MBS266, respectively, and grow cultures overnight (ON) for at least 12 hours, at 30 °C with constant shaking at 250 rpm. For the step 2 of the analysis, inoculate 100 ml of SD “light” and SD “heavy” media with fresh colonies from either MBS402 (or?) and MBS266, respectively (*see* Note 9).
2. On the next day the cultures OD₆₀₀ should be around 0.4 – 0.5. Dilute cultures in their respective SD media to an OD₆₀₀ = 0.1 in a final volume of 250 ml (*see* Note 10).

3.2. Methyl Methanesulfonate (MMS)-Induced Replication Stress and Cells Harvesting

Previous work has shown that methyl methanesulfonate (MMS) concentrations up to 0.04 % are enough to efficiently activate the DNA damage checkpoint response during S-phase but not during G1 [19]. For the purpose of this protocol we are using 0.04 % of MMS to address checkpoint-dependent Rtt107 protein-protein interactions during S-phase (*see* Note 11).

1. When cells reach an OD₆₀₀ = 0.4, add 100 µl of MMS to final concentration of 0.04 % and keep cultures growing for 2.5 hours.
2. After 2.5 hours treatment with MMS transfer the 2 × 250 ml cultures to 2 separated 500 ml conical centrifugation bottles.
3. Centrifuge the cultures for 2,500 × g for 5 min at 4 °C. Discard the supernatant.

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⁹Use fresh yeast culture plates.

¹⁰Avoid culture saturation in SD media (OD₆₀₀ > 0.5). Once saturated SILAC strains won't grow well. If culture is saturated, redilute and let it grow for at least 4 generations before proceeding with the experiment.

¹¹MMS is extremely toxic and should be handle in a chemical fume hood. All glasses and bottles in contact with MMS should be washed with a 10 % sodium thiosulfate solution. Cell culture medium containing MMS should be inactivated by adding an equal volume of 10 % sodium thiosulfate.

4. Add 10 ml of ice-cold TE buffer to each centrifugation bottle, resuspend cell pellets and transfer each pellet to a separate 15 ml Falcon tubes
5. Repeat step from section 3.2.3.
6. Add 2 ml of ice-cold TE buffer to each 15 ml Falcon tube, resuspend each cell pellet separately and transfer each pellet to 2×2 ml conical screw cap tube. Spin down cells for 15 s in a bench top microcentrifuge for $5,000 \times g$ at room temperature (RT) and remove supernatant. Each cell pellet should be around 150–200 mg. Cell pellets can be stored at -80°C for up to 2 weeks.

3.3. Cell Lysis

1. Add 600 μl of ice-cold glass beads to each tube.
2. Add 1 ml of lysis buffer to each tube and break cells for 15 min at 4°C using the bead beater.
3. Puncture the bottom of the 2 ml tube using a hot 21-gauge needle. Place tube on top of a 5 ml syringe and place the syringe inside a 15 ml Falcon tube. Centrifuge at 1000 rpm at 4°C for 3 min to recover extract.
4. Transfer lysate to a 1.7 ml microcentrifuge and centrifuge for 10 min at $13,000 \times g$ at 4°C .
5. Collect the cleared supernatant and determine the protein concentration of both samples by using protein assay dye reagent.
6. Normalize protein concentration between the two samples (MBS164 “Light” and MBS266 “Heavy” or MBS402 “light” and MBS 266 “heavy”). Final concentration should range between 7.5–10 mg/ml and total amount of protein should be around 15–20 mg. After normalization, dilute samples to maximum concentration of 5 mg/ml (final volume of 3–4 ml) and transfer each sample to a different 15 ml Falcon tube. Keep samples on ice.

3.4. Immunoprecipitation of 3xHA-tagged Rtt107

1. Condition anti-HA resin by washing $3 \times$ in at least 500 μl of lysis buffer. Use a minimum of 50 μl of anti-HA resin per 15–20 mg of protein. Add the anti-HA resin to the samples and incubate for 2–4 hours at 4°C under constant agitation (*see* Note 12).
2. Collect resin by quick spin at 4°C for 1 min at $2,500 \times g$ and wash resin $3 \times$ with at least 500 μl of ice-cold lysis buffer (*see* Note 13).
3. Add 3 volumes of elution buffer to the resin and heat it at 95°C for 5 min (*see* Note 14).

¹²This is the starting point for depletion of the Rtt107-3xHA. For other proteins the amount of HA resin and time of incubation should be empirically tested.

¹³Washing repetitions should be empirically tested.

¹⁴If too much IgG background is detected during the MS analysis, stop adding DTT to the elution buffer. In this case, add DTT to the sample after elution from beads.

4. Transfer the resin and elution buffer to a Micro Bio-Spin® column pre-equilibrated with elution buffer. Collect elution in a 1.7 ml microcentrifuge tube by spinning the column at $650 \times g$ for 1 min at RT.

3.5. Protein Precipitation and Tryptic Digestion

1. At this point combine (all?) elutions from immunoprecipitations in a 1.7 ml microcentrifuge tube.
2. For protein alkylation, add 15 μ l of a 0.5 M iodoacetamide stock solution (final concentration of 25 mM) and incubate for 15 min at RT (*see* Note 5).
3. Use a speed-vac to concentrate sample at 45 °C until it reaches 100 μ l (one third of the initial volume) (*see* Note 15).
4. Add 300 μ l of protein precipitation solution and incubate sample on ice for 30 min.
5. Centrifuge sample at 13,000 g for 10 min at RT.
6. Discard supernatant carefully without dislodging the tiny protein pellet.
7. Add 200 μ l of protein precipitation solution and sonicate sample in water bath sonicator for 10 seconds (*see* Note 16).
8. Repeat step from sections 3.5.5 and 3.5.6.
9. Keep microcentrifuge tube upside-down for 5–10 min until acetone smell is gone.
10. Resuspend protein pellet in 50 μ l of Urea/Tris solution by carefully pipeting up and down (*see* Note 5).
11. Add 150 μ l NaCl₂/Tris solution.
12. Add 1 μ g of Gold Trypsin®, seal microcentrifuge tube with parafilm and incubate sample ON at 37 °C.

3.6. Protein Sample Clean-Up

1. Using a glass syringe, acidify sample by adding 5 μ l of 10 % trifluoroacetic acid and 5 μ l of 10 % formic acid (0.25 % final concentration each).
2. Spin sample at $13,000 \times g$ for 1 minute at room temperature to remove particulates prior sample clean-up. Transfer supernatant to a new microcentrifuge tube.
3. By applying air pressure with a pipette bulb, condition a 50 mg Sep-Pak® C₁₈ column by adding 1 ml of C₁₈ buffer D (*see* Note 17).
4. Add 1 ml of C₁₈ buffer A to equilibrate column.
5. Apply the sample through the column and let it flow by gravity.
6. Add 2×1 ml of C₁₈ buffer B to wash the column. Let it flow by gravity.

¹⁵This step is required to increase the efficiency of protein precipitation.

¹⁶Wash the walls of the microcentrifuge tube carefully to remove all traces of detergent.

¹⁷Avoid letting the C₁₈ columns to dry.

7. Wipe residual volume of C₁₈ buffer C off column tip using a Kimwipe®.
8. By applying air pressure with a bulb, elute sample in 200 µl of C₁₈ buffer C in a polyspring® silanized conical base insert vial.
9. Using a gel loader tip, mix sample by pipeting up and down.
10. Dry sample completely at 45 °C using a speed-vac concentrator.
11. To resuspend sample, add 15 µl of distilled water.
12. By using a glass syringe, add 15 µl of 10 % formic acid and 60 µl of 99.9% acetonitrile to sample. Using a gel loading tip, mix sample by pipeting up and down (*see* Note 18).
13. Transfer the vial to 1.7 ml microcentrifuge tube and spin it at 5,000 × g for 1 minute at room temperature to remove particulates prior HILIC chromatography.
14. Transfer sample (approximately 85 µl) to another polyspring® silanized conical base insert vial using a gel loader tip.

3.7. Hydrophilic Interaction Liquid Chromatography (HILIC)

The unique separation ability of Hydrophilic Interaction Liquid Chromatography (HILIC) and its orthogonality towards other reverse phase separation techniques make it an ideal method for multidimensional fractionation of complex protein samples prior to MS analysis [20]. The present study was performed using a Dionex Ultimate 3000 HPLC controlled by Chromeleon 6.8 software. A TSK gel Amide-80 column (2 mm × 150 mm, 5 µm; Tosoh Bioscience) was used for the HILIC fractionation experiment. Three buffers were used for the gradient: buffer D (90 % acetonitrile); buffer E (80 % acetonitrile and 0.005 % trifluoroacetic acid) and buffer F (0.025 % trifluoroacetic acid).

1. Load samples into HILIC TSK gel Amide-80 column via a 100 µl loop and set the fraction collector to time-based mode, collecting 1 min fractions between 10 and 22 min of the gradient in 300 µl polyspring® silanized conical base insert vials. The gradient used consists of 100 % buffer D at time = 0 min, 98 % of buffer E and 2 % of buffer F at time = 5 min, 82 % of buffer E and 18 % of buffer F at time = 15 min and 5 % of buffer E and 95 % of buffer F from time = 25 to 27 min in a flow of 150 µl/min.
2. Dry sample fractions completely at 45 °C using a speed-vac concentrator.
3. Resuspend each fraction in 7 µl of 0.1 % trifluoroacetic acid.

3.8. Reverse Phase Liquid Chromatography-Tandem Mass Spectrometry

As part of our multidimensional separation platform, we perform a C₁₈ reverse phase fractionation prior to MS analysis. C₁₈ reverse phase is a high resolution fractionation technique that presents an orthogonal selectivity towards HILIC [20]. The present study was

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¹⁸If sample won't be fractionated immediately, it should be resuspended in 15 µl of water and kept at -80 °C. Add the 10 µl of 10 % formic acid and 60 µl of 99.9% acetonitrile right before HILIC fractionation.

performed by using a on-line Nano LC-Ultra® system (Eksigent) coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific).

1. Load sample fractions into a 20-cm column with 125 μm inner diameter, packed in-house with 3 μm C₁₈ particles. Reverse phase chromatography is performed with a binary buffer system consisting of buffer I (0.1 % formic acid) and buffer II (0.1 % formic acid with 80 % acetonitrile). The peptides are separated by a linear gradient of buffer II up to 95 % for 80 min with a flow rate of 200 nl/min.
2. Xcalibur 2.2 software (Thermo Fischer Scientific) was used for the data acquisition and the Q Exactive [21] was operated in the data-dependent mode. Survey scans were acquired in the Orbitrap mass analyzer over the range of 380 to 2000 m/z with a mass resolution of 70.000 (at m/z 200). The maximum ion injection time for the survey scan was 80ms with a 3e8 automatic gain-control target ions. MS/MS spectra was performed selecting up to the 10 most abundant isotopes with a charge state $\geq$ 2 within an isolation window of 2.0 m/z. Selected isotopes were fragmented by higher energy collisional dissociation with normalized collision energies of 27 and the tandem mass spectra was acquired in the Orbitrap mass analyzer with a mass resolution of 17.500 (at m/z 200). The maximum ion injection time for the MS/MS scans were 120 ms with a 1e5 automatic gain-control target ions. Repeat sequencing of peptides was kept to a minimum by dynamic exclusion of the sequenced peptides for 30 seconds.

3.9. Database Searching and Protein Quantification

Raw MS/MS spectra was searched by using SEQUEST software on a SORCERER system (from Sage-N Research) using a composite yeast protein database, consisting of both the normal yeast protein sequences and their reversed protein sequences as a decoy to estimate the false discovery rate in the search results.

1. The following parameters were used in the database search: Semi-tryptic requirement, a mass accuracy of 15 ppm for the precursor ions, differential modification of 8.0142 Daltons for lysine and 10.00827 Daltons for arginine. Finally, results were filtered based on Probability Score to achieve a 1 % false positive rate.
2. XPRESS software was used to quantify all the identified peptides.
3. In the first step of the analysis the specific interacting partners of Rtt107-3xHA are identified by abundance ratios significantly higher than 1 while co-purified background proteins exhibit abundance ratios of around 1 (*see* Fig. 2A). In the second step of the analysis the Mec1-dependent interacting partners of Rtt107-3xHA are identified by abundance ratios significantly higher than 1 while Mec1-independent interactions exhibit abundance ratios of around 1 (*see* Fig. 2B).

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Fig. 1.

Overview of the methodology for the identification of checkpoint-dependent interactions of Rtt107-3xHA during replication stress. (a) Untagged and 3xHA tagged Rtt107 strains were grown in “Light” and “Heavy” media, respectively. After cultures were treated with 0.04% methyl-methanesulfonate (MMS), proteins were extracted and subjected to anti-HA immunoprecipitation (IP). Immunoprecipitation elutions were mixed, fractionated by HILIC and subjected to LC - quantitative mass spectrometry analysis. Peptides ratio significantly higher than 1 indicates Rtt107 specific interactions while peptides ratio around 1 indicates

nonspecific interactions. (b) *mec1* and wild type cells, both containing Rtt107-3xHA were grown in "light" and "heavy" media, respectively. After LC - quantitative mass spectrometry analysis, only the proteins previously identified as Rtt107 specific interactors were considered. Heavy-to-light peptide ratio significantly higher than 1 indicates checkpoint-dependent Rtt107 interactions while heavy-to-light peptide ratio around 1 indicates checkpoint-independent Rtt107 interactions. Proteins 1 to 11 were identified with a heavy-to-light ratio greater than 10 and considered to be Rtt107 specific interacting protein.

Fig. 2.

Results from experiments depicted in Figure 1. (a) Ratios of Rtt107-3xHA IP (heavy) / Rtt107 untagged IP (light) for the 1243 proteins identified. Each ratio corresponds to the geometric mean of the ratios from all peptides identified and quantified for each protein. Eleven proteins with ratios greater than 10 were considered to be specific Rtt107-interacting proteins. (b) Ratios of *mec1* IP (light) / wt IP (heavy) for the specific Rtt107-interacting proteins identified. Ratio for each protein was calculated by averaging the ratios of all corresponding peptides identified and quantified in the analysis. The protein Dpb11,

assigned here as protein 3 (in red), was identified with a heavy-to-light ratio greater than 10 and considered to be a Mec1-dependent Rtt107-interacting protein.