Robust co-immunoprecipitation with mass spectrometry for Caenorhabditis elegans using solid-phase enhanced sample preparation

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ABSTRACT
Studying protein interactions in vivo can reveal key molecular mechanisms of biological processes. Co-immunoprecipitation with mass spectrometry detects protein–protein interactions with high throughput. The nematode Caenorhabditis elegans is a powerful genetic model organism for in vivo studies. Yet its rigid and complex tissues require optimization for biochemistry applications to ensure reproducibility. The authors optimized co-immunoprecipitation with mass spectrometry by combining a native co-immunoprecipitation procedure with single-pot, solid-phase enhanced sample preparation. The authors’ results for the highly conserved chromatin regulator FACT subunits HMG-3 and HMG-4 demonstrated that single-pot, solid-phase enhanced sample preparation-integrated co-immunoprecipitation with mass spectrometry procedures for C. elegans samples are highly robust. Moreover, in an accompanying study about the chromodomain factor MRG-1 (MRG15 in humans), the authors demonstrated remarkably high reproducibility for ten replicate experiments.

METHOD SUMMARY
A combination of cryofracture and single-pot, solid-phase enhanced sample preparation for the performance of co-immunoprecipitation with mass spectrometry provides robust assessments of protein–protein interactions using Caenorhabditis elegans whole animals.

TWEETABLE ABSTRACT

KEYWORDS:
C. elegans ● co-immunoprecipitation ● FACT ● HMG-3 ● HMG-4 ● mass spectrometry ● protein–protein interaction ● SP3

In living organisms, proteins are essential components of cellular structures and transport machinery and perform vital enzymatic reactions during biochemical processes. Furthermore, proteins have central functions for gene expression and DNA maintenance. Therefore, studying protein–protein interactions is important for understanding the vast array of molecular mechanisms and biochemical pathways in living cells.

Although in vitro applications such as protein pull-downs indicate potential interactions, the detection of protein–protein interactions directly from cells is critical for obtaining relevant insight into actual protein–protein interaction networks. The application of co-immunoprecipitation with mass spectrometry (CoIP-MS) allows detection of in vivo protein–protein interactions. However, because of various factors that depend on the type of research model used, co-immunoprecipitation (CoIP) from multicellular organisms is not straightforward.

The nematode Caenorhabditis elegans is a powerful genetic model organism. However, its rigid cuticle and complex tissues require optimization for protein biochemistry applications to ensure reproducibility of experimental outcomes. Therefore, the authors optimized the application of CoIP-MS to C. elegans by combining a native CoIP procedure with an efficient sample preparation technique called single-pot, solid-phase enhanced sample preparation (SP3) [1,2].

Standard native CoIP protocols for C. elegans involve physical and chemical shearing to break up the tough cuticle layer. For physical shearing, an instant freeze step in liquid nitrogen preserves protein–protein interactions during subsequent cryofracture of the animals. Cryofracture makes tissues accessible for buffers containing required chemicals such as detergents to solubilize proteins prior to mass spectrometry (MS) analysis [3]. However, detergents and other components of lysis buffers that need to be used for the lysis of rigid C. elegans tissues strongly interfere with MS analysis, resulting in reduced reproducibility of experiments [4]. After proteins are released from
the tissues, a sonication step is necessary for the fragmentation of viscous DNA to prevent interference with the target protein's precipitation. Removing excessive DNA is especially important when purifying chromatin-regulating proteins because unspecific interactions could be mediated via genomic DNA. To distinguish specific interactions from unspecific binding proteins, which can cause significant background and noise, the immunoprecipitated samples are compared with a proper negative control [5]. Nonspecific background contaminants can be caused either by the affinity of unspecific proteins for the solid matrices used to precipitate the target protein or by cross-reactivities of antibodies. Having unique and efficient antibodies for the target protein of choice is not always feasible but can be bypassed by fusing epitope tags such as HA or FLAG to the target protein [6]. Some commercially available antibodies against such epitopes provide high-affinity binding, allowing stringent washing procedures during purification steps to remove background binders. Additionally, magnetic beads that are already coupled to, for example, anti-HA antibodies or protein A/G with high affinities for primary antibodies (immunoglobulins) allow magnetic separation of the target protein and its interacting proteins in a highly efficient and specific manner. Again, strong detergents and denaturing conditions need to be used to elute the target protein from the beads. However, as mentioned earlier, detergents are problematic, as they are incompatible with the enzymes used for proteolysis in bottom-up proteomics and MS analysis. Therefore, various methods, including ultrafiltration [7] and precipitation [8], have been utilized to purify protein samples prior to MS analysis. Yet these methods have shortcomings, as they usually require relatively high amounts of sample and are therefore not suited for high-throughput sample preparations. To address this, a technique known as SP3 was developed as a rapid and efficient method of purifying protein that is compatible with a range of chemicals without being restricted to high-input material [1,9]. SP3 makes use of carboxylated magnetic beads with a hydrophilic surface to confine proteins and peptides in a manner similar to that obtained with hydrophilic interaction liquid chromatography [10]. In addition, aggregation of insoluble proteins on carboxylated beads under high organic solvent conditions has been demonstrated as a binding mechanism [11]. Proteins trapped on the beads can then be washed vigorously to eliminate contaminants, detergents and salts that interfere with MS.

To improve CoIP-MS analysis of protein–protein interactions in whole animal lysates of C. elegans, which contain a number of strong detergents and salts, the authors established a CoIP-MS working pipeline with integrated SP3 application. Additionally, the authors tested the effects of enzymes that remove DNA and RNA, which can cause background. Based on the subunits HMG-3 and HMG-4 of the heteromeric chromatin remodeler FACT [12–14], the authors demonstrated that the SP3-integrated CoIP-MS procedure for C. elegans samples is highly accurate and robust.

Methods

Worm strains
The wild-type C. elegans Bristol strain N2 and CRISPR strains were maintained according to the standard protocol at 20 °C. The strains used in this study were: BAT1753 hmg-3 (bar24[hmg-3::3xHA]) I, (CRISPR/Cas9), BAT1954 hmg-4 (bar30[hmg-4::3xHA]) III (CRISPR/Cas9), wild-type N2.

Synchronized worm population
Synchronized worms were obtained by standard bleaching procedure using sodium hypochlorite solution to disintegrate gravid adult worms as previously described [14]. Briefly, 5% sodium hypochlorite solution was mixed with 1 M NaOH and water in a 3:2:5 ratio. M9 buffer was applied to wash the worms off nematode growth medium plates. Worms in M9 buffer were mixed with bleaching solution for 5 min in a 1:1 ratio and vortexed until the adults started dissolving. To remove bleach solution completely, released embryos were washed three times with M9 buffer. After overnight incubation, synchronized L1 stage worms were obtained. L1s were applied directly onto regular nematode growth medium plates for further maintenance of a synchronized population.

Western blot
Input and CoIP samples were frozen at -20 °C. Right before loading, SDS-PAGE sample buffer was added. Samples were boiled for 10 min to denature the proteins and centrifuged for 10 min at full speed. HMG-3::3xHA and HMG-4::3xHA were detected with anti-HMG-3/-4 antibody (Pineda, Berlin, Germany) at a dilution of 1:1000.

Antibodies & affinity matrix
The authors used anti-HMG-3/-4 antibody (Pineda, Berlin, Germany), ChIP-grade anti-HA antibody (ab9110; Abcam, Cambridge, UK), magnetic carboxylate-modified Sera-Mag A beads (category no. 09-981-121; Thermo Fisher Scientific, MA, USA) and magnetic carboxylate-modified Sera-Mag B beads (category no. 09-981-123; Thermo Fisher Scientific).

Immunoprecipitation with MS
For each condition, three biological replicates were collected as 300 μl of L4/young adult stage worm pellet. Wild-type, HMG-3::3xHA and HMG-4::3xHA worms were collected in M9 buffer, washed four times with M9 to remove bacteria and concentrated into a worm pellet after the last wash. The worm pellet was added dropwise to liquid nitrogen, paying special attention to be sure that the resulting ‘worm beads’ were around the size of black pepper to ensure even grinding afterward. With the help of a pulverizer, the frozen worms were then cryofractured. To achieve even grinding of all tissues, worms were further ground using a mortar and pestle on dry ice. The worm powder
was mixed with 1.5× lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM MgCl2, 0.1% Tween 20 and protease inhibitors), dounced with a tight douncer 30 times and sonicated using a Bioruptor (Diagenode, USA, MA) with high setting (30 s on and 30 s off a total of six times). The resulting worm lysis was centrifuged at 16,000 × g at 4ºC for 10 min to remove the insoluble pellet. The supernatant was transferred to 2-ml Eppendorf (Hamburg, Germany) tubes. The protein concentration of each worm lysis for each biological replicate was determined by Bradford assay and set to 2 mg/ml. ChIP-grade anti-HA antibody was then added to the samples to incubate for 30 min on a rotator at 4ºC. Next, μMACS Protein A beads (Miltenyi Biotec, North Rhine-Westphalia, Germany) were added to the samples as instructed in the kit, and samples were incubated for 30 min on a rotator at 4ºC [15]. Meanwhile, the μMACS columns were placed on a magnetic separator to be equilibrated and ready for sample application. Samples were diluted to 5× their volume with lysis buffer, adding up to 10 ml before being applied to columns, and the columns with bound proteins were washed three times with lysis buffer to remove background binders. The proteins were eluted with elution buffer (100 mM Tris-Cl [pH 6.8], 4% SDS, 20 mM DTT) and heated to 95ºC. Eluted samples were prepared for MS measurements by SP3 [1,2]. After the final elution step, the amount of protein in CoIP samples in SDS buffer was determined by a detergent-compatible assay that was compatible with SDS to a final amount of 50 μg/μl before SP3 cleanup.

**SP3 method**

Magnetic carboxylate-modified Sera-Mag A and Sera-Mag B beads were brought to room temperature for 10 min. A volume of 20 μl Sera-Mag A beads was combined with 20 μl of Sera-Mag B beads and washed with 160 μl of water by placing the water–bead mixture on a magnetic rack for PCR tubes (DynaMag-PCR magnet, category no. 492025, Thermo Fisher Scientific), and beads were settled for 2 min. Magnetic beads were rinsed with 200 μl of LC-MS-grade water (Thermo Fisher Scientific) by pipette mixing (off the magnetic stand); this was repeated two additional times. The final bead pellet was stored in 100 μl of water in the refrigerator until use. A CoIP sample in the amount of 50 μg was transferred to a PCR tube and incubated with 1 μl of DNase or Benzonase (category no. E8263; Sigma-Aldrich, MO, USA) at 37ºC for 30 min to shear and digest nucleic acids. Benzonase is an endonuclease from *Serratia marcescens* that can degrade all types of DNA and RNA but has no proteolytic activity. A volume of 10 μl of 50 mM TCEP in 50 mM ABC was added for reduction and incubated at 25ºC for 20 min. A total of 10 μl of 400 mM CAA in 50 mM ABC was added for alkylation of the samples and incubated at 25ºC for 30 min in the dark. Next, 5 μl of the bead stock was added to each sample. For buffer exchange, acetonitrile was added to a final percentage of 50% (v/v) and incubated for 10 min off the magnetic rack while vortexing with Vortex Genie (Scientific Industries, Inc., NY, USA) at level 4 with 1-min intervals, avoiding spillovers from one tube to another. The samples were then incubated on a magnetic rack for 2 min, and the supernatant was discarded. Next, 80% (v/v) 200 μl ethanol was added, and the samples were incubated off the magnetic rack for 30 s. The samples were then incubated on a magnetic rack for 2 min, and the supernatant was discarded. Another round of washing step was carried out. A total of 180 μl acetonitrile was added and incubated for 15 s off the magnetic rack followed by 2-min incubation on a magnetic rack. The supernatant was discarded, and the samples were air-dried in a safety hood.

The beads were reconstituted in 5 μl digestion solution consisting of 50 mM HEPES plus Trypsin/Lys-C mix (1:2.5 enzyme-to-substrate ratio with 1 μg trypsin and 1 μg Lys-C per sample) and incubated for 14–16 h at 37ºC (PCR machine with 80ºC heated lid). For peptide recovery, tubes were placed on the magnetic rack for 2 min, and the supernatant was collected in fresh tubes. A total of 40 μl 50 mM HEPES was added to the beads, which were resuspended, sonicated for 3 min and placed on a magnetic rack. The supernatant was collected and combined with the first supernatant. The sample was acidified with TFA to a final concentration of 1% (v/v). To remove salts and contamination, stage tips with two layers of C18 were prepared as described previously [16]. To condition the stage tips, 100 μl MEOH, 100 μl 50% (v/v) acetonitrile and 100 μl 0.1% (v/v) formic acid were used. The samples were then loaded to the stage tips followed by washes with 100 μl 2% ACN, 100 μl 1% TFA and 100 μl 1% FA. The stage tips were either stored at 4ºC until use or eluted immediately with 50% acetonitrile and 0.1% FA. The peptides were concentrated and dried using a SpeedVac (Thermo Fisher Scientific) at 35ºC for approximately 30 min. Concentrated peptides were resuspended in 5 μl 0.1% TFA, and a volume of 2 μl was used for injection into LC-MS.

**LC-MS/MS analysis**

Samples were measured with 1-h gradient at 15K resolution and 100-ms injection time by LC-MS/MS on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) connected to an EASY-nLC system (Thermo Fisher Scientific). The samples were separated on a 44-mm gradient, ramping from 5 to 55% acetonitrile, using an in-house-prepared nano-liquid chromatography column (0.074×250-mm, 3-μm ReproSil C18, Dr Maisch HPLC GmbH, Ammerbuch, Germany) and a flow rate of 250 nl/min. MS acquisition was operated at an MS1 resolution of 70,000 and a scan range of 300–1700 m/z. For data-dependent MS2 acquisition, the top ten peaks were selected for MS2 with a resolution of 17,500, maximum injection time of 60 ms and isolation window of 2 m/z. Automatic gain control target was set to 2.5e3, and dynamic exclusion was specified at 20 s.

**MS data analysis**

Raw data files were processed with default settings (unless stated otherwise) in MaxQuant 1.5.2.8. [17]. Protein quantification was performed using the label-free quantification (LFQ) MaxLFQ algorithm [18]. The ’match between runs’ option was chosen for transferring MS/MS identifications between LC-MS/MS runs. Trypsin/P enzyme was used at the specific enzyme setting. Cysteine carbamidomethylation was used as fixed modification, and oxidation of methionine and acetylation of the protein N terminus were set as variable modifi-
Results

General method overview

Interaction proteomics involves detecting the specific interactors of a particular protein when those interactors are significantly enriched. For this purpose, label-free purification methods provide sufficient robustness with the implementation of the LFQ algorithm in MaxQuant software [18]. When label-free approaches such as MaxLFQ were compared with metabolic labeling, MaxLFQ proved to be as accurate as, for example, SILAC [20]. Overall, LFQ analysis is suitable for interaction proteomics, but accurate assessment of individual protein ratios in LFQ requires a t-test with three or more replicates [18]. Therefore, the authors performed at least three biological replicates for each bait protein. During method optimization, manual cryo-fracturing was performed using a mortar and pestle (Figure 1).

For cryo-fracturing, worms were first frozen in liquid nitrogen as pellets and subsequently powdered using hammering and grinding followed by cell lysis and sonication (Figure 1). For the authors’ endogenously expressed 3xHA-tagged proteins (derived by CRISPR/Cas9 gene editing), samples were incubated with anti-HA antibodies followed by protein A, which were coupled to magnetic beads and therefore allowed efficient separation of the beads from bound protein using magnetic racks. After eluting the proteins off the immunoprecipitation beads using an SDS- and DTT-containing elution buffer, the protein fraction was further cleaned up using SP3 [1,2]. The authors rationalized that cleaning up samples before MS analysis with SP3 would be beneficial because previous CoIP-MS results without removing detergent and salts using solid-phase enhanced purification resulted in low correlation of replicate experiments (Supplementary Figure 1A). By contrast, the application of SP3 provided strong correlation of replicate experiments. The authors also demonstrated a remarkable robustness of repeat experiments in a parallel study, showing the reproducibility of ten replicate experiments (Supplementary Figure 1B) [21].

SP3 makes use of carboxylated magnetic beads with a hydrophilic surface to bind proteins and peptides, thereby allowing vigorous washing steps to eliminate contaminants, detergents and salts that interfere with MS. Another aspect of removing noise-causing cell components prior to performing immunoprecipitation is removing DNA and RNA. Nucleic acids can cause artificial interaction of DNA- and RNA-binding proteins by bridging two proteins that bind nucleic acids but do not interact directly with each other [22]. DNA and RNA can be eliminated from cell lysates by applying enzymes such as DNase and Benzonase, which cleave all DNA and RNA. However, it is not clear whether their use can interfere with CoIP-MS. Therefore, the authors assessed, as described in the following section, their effect on CoIP-MS. The authors used whole worm samples and targeted the chromatin-binding proteins HMG-3 and HMG-4, which was previously tagged with 3xHA using CRISPR/Cas9 [14].

The effect of Benzonase & DNase on the interactors of chromatin regulator HMG-3

CoIPs were performed separately with or without DNase and Benzonase treatment. Benzonase was chosen as a more efficient enzyme compared with DNase and for its ability to chop down both DNA and RNA molecules at 4°C. To check the reaction efficiency of both enzymes at 4°C, which is the temperature at which immunoprecipitation is carried out, 5 μg gDNA was incubated with each enzyme and compared with a longer reaction period at 4°C (Figure 2A). Benzonase showed higher efficiency by digesting all of the gDNA at 4°C. Although there was gDNA detectable after DNase application, less DNA was observed after 16 h at 4°C compared with that seen under optimal reaction conditions with DNase and was an indication of the enzyme’s activity at 4°C. To compare the impact of Benzonase and DNase treatment on chromatin regulator interactions, protein–protein interactions of the germline-specific chromatin regulator HMG-3 were compared between enzyme treatments and the control. In the control case, 41 proteins appeared to be interacting with HMG-3 significantly (Figure 2B). Gene Ontology analysis based on WormBase Enrichment analysis (https://wormbase.org/tools/enrichment) revealed unexpected enrichments, such as for actin-binding and actin filament-based processes (Figure 2C). This might be due to HMG-3’s nonspecific interactions with structural proteins as a result of the presence of DNA. Upon DNase treatment (Figure 2D), Gene Ontology term analysis of the significant interactors of HMG-3 showed the expected involvement in more chromatin-based and germline-specific functions such as reproduction (Figure 2E). The elimination of DNA-directed, nonspecific interactions of HMG-3 upon DNase treatment could account for a reduction in background interactions. In addition, Benzonase-applied samples showed different enrichment of protein interactions (Figure 2F). Even though the analyzed protein amounts were the same in all samples, the number of interaction partners and overall identified proteins dropped significantly for Benzonase-treated samples. This might be due to either the higher potency of Benzonase or the degradation of RNA in addition to DNA upon Benzonase application (Figure 2G).

FACT subunits also directly interacted with RNA to stabilize the heterodimer [24]. Hence, degradation of RNAs may decrease their interaction potential with other factors when FACT subunit association is decreased such that less interactions are detected (Figure 2D, F & H). Generally, the presence of RNA is thought to be relevant for interactions with many other nuclear- and chromatin-associated factors such that the presence of RNA may be relevant for detecting interactions of nuclear proteins [25].

Although Benzonase- and DNase-treated samples correlated well with regard to overall detected proteins (Supplementary Figure 2), treatments with Benzonase and DNase can alter the detection of significantly enriched proteins (Figure 2H). Therefore, researchers should consider applying both treatments if no information about the tested proteins is available and compare the results. In this case, the authors chose DNase treatment to preserve potentially relevant FACT subunit interactions that may depend on the presence of RNA.
Figure 1. Workflow of native co-immunoprecipitation with mass spectrometry for *Caenorhabditis elegans* lysates with single-pot, solid-phase enhanced sample preparation.
Figure 2. Impact of DNase and Benzonase on detectable interactions of the chromatin regulator HMG-3. CoIP-MS was used to assess protein–protein interactions of HMG-3. (A) Agarose gel electrophoresis detection of 5 μg gDNA incubated with DNase and Benzonase at 37 °C and 4 °C for 30 min and 16 h, respectively. Wild-type N2 was used with anti-HMG-3 and nonspecific antibodies were used as controls. (B, D & F) Volcano plots showing statistically significant enrichment of co-precipitated proteins as measured by t-test. Adjusted p-value is set as indicated on each plot as false discovery rate cutoff. (C, E & G) Gene set enrichment analysis of proteins interacting with HMG-3 using WormBase WS279. (H) Venn diagram showing overlaps of enriched proteins with different treatments.

CoIP-MS: Co-immunoprecipitation with mass spectrometry.
Venn diagram generated using InteractiVenn [23].
Testing the specificity & cross-reactivity of anti-HA antibody for CoIP-MS

In several affinity purification studies, large epitope tags were shown to be more likely to affect the function of proteins by changing their conformation and folding [26]. Moreover, CRISPR knock-in of fluorescent proteins is known to be less efficient than smaller tags such as HA [27].

With the goal of assessing the efficiency of small affinity purification based on the HA tag, the authors made use of strains tagged with 3xHA at the C-terminus of FACT complex members HMG-3 and HMG-4 (Figure 3). FACT is a chromatin regulator that is identified as a reprogramming barrier in both C. elegans and humans [14]. FACT’s interaction partners have not been previously analyzed in C. elegans. FACT, which is composed of SSRP1/SUPT16H in humans [12–14], forms two different complexes based on the tissue type in C. elegans [14]. FACT is composed of HMG-3 and SPT-16 in the germline, whereas HMG-4 is mostly absent in the germline. In somatic tissues, HMG-3 is completely absent and substituted by HMG-4 to form the heterodimer with SPT-16. Keeping in mind the tissue specificity of the FACT heterodimers in C. elegans, immunoprecipitating HMG-3 and HMG-4 with the same antibody to compare their interaction partners was an ideal setup to evaluate the efficiency of the authors’ CoIP-MS protocol.

To assess the robustness of the CoIP protocol, CRISPR-edited animals carrying HMG-3::3xHA, HMG-4::3xHA and wild-type N2 worms were prepared (Figure 3A). In the pull-down assays against HMG-3::3xHA and HMG-4::3xHA, 200 μl of worm pellet from young gravid hermaphrodites was collected and snap frozen in liquid nitrogen. Three biological replicates were prepared in parallel for each strain. The same anti-HA antibody was used with wild-type N2 worms to detect nonspecific binders of the antibody.

CoIPs were prepared, and the abundance of co-purified proteins was assessed by label-free quantitative MS to identify proteins specifically enriched in the HMG-3::3xHA and HMG-4::3xHA pull-downs (Figure 3A). The most significant interactor for both proteins was their heterodimer partner, SPT-16 [12–14], indicating the robustness of the CoIPs (Figure 3B–E). Notably, tissue ontology terms for significantly enriched proteins for both HMG-3 and HMG-4 showed differential tissue expression, as expected. Although HMG-3-enriched proteins were correlated exclusively with the germline, HMG-4 interacting proteins were correlated with gonadal primordium and somatic cells such as neurons (Figure 3C & E). A total of 12 proteins were identified as interacting with both HMG-3 and HMG-4 (Figure 3F). Overall, these results revealed previously unknown potential interaction partners of FACT, demonstrating the high efficiency of the authors’ CoIP-MS method achieved by maintaining the tissue-specific interaction partners of HMG-3 and HMG-4.

Discussion & conclusion

Physiological processes in living organisms depend on protein interactions. Hence, studying the in vivo interactome of proteins is required to expand our knowledge of protein dynamics derived from investigating cell lines outside of their physiological environments [28,29]. To establish a reliable method for studying protein interactions in a multicellular organism, the authors used the nematode C. elegans. Although MS for protein interaction studies has been conducted in C. elegans previously [3,30,31], its application in worms is not straightforward. Proper lysis of all tissues is difficult because of the cuticle, and the content of the entire worm body, such as fat and other organic compounds, can severely confound reproducibility.

In this study, the authors are providing a highly reproducible and robust protocol for CoIP-MS. The authors combined a native CoIP protocol for worm lysates with the SP3 method [1,2,9]. The authors introduced the 3xHA tag, which can be targeted using commercially available anti-HA antibodies in a highly efficient manner by CRISPR to two proteins of interest: HMG-3 and HMG-4. In a parallel study, which is reported in an accompanying article [21], the authors also tagged the chromatin regulator MRG-1 with 3xHA using CRISPR editing. Wild-type animals without the 3xHA knock-in can be used as controls to eliminate background binders of the antibody as well as purification resin. In order to have confidence in the data, three replicate experiments were performed for HMG-3 and HMG-4, whereas in the accompanying study, at least ten replicates were performed for MRG-1. The authors also evaluated the effects of Benzonase and DNase treatment of the protein lysate sample preparation. Both enzymes can be used to remove nucleic acids, as these can cause background protein interactions of, for instance, chromatin-binding proteins such as HMG-3 and HMG-4. Interestingly, the number of protein interactions and intensities of detected peptides for HMG-3 was significantly less for Benzonase-treated samples. Although it remains to be understood whether this effect is due to the activity of Benzonase on RNA, the authors decided to apply DNase for further analysis since many functionally relevant interactions of chromatin-binding proteins also depend on the presence of RNA and DNA. DNase treatment may preserve potentially relevant interactions that may become otherwise undetectable as a result of decreased peptide intensities upon treatment with Benzonase.

To measure the intensities of the precipitated proteins, the authors used LFQ. Although the detected peptides are utilized to deduce the identity and quantity of proteins in bottom-up proteomics, MS measurements are not inherently quantitative [32]. In bottom-up proteomics, mass spectrometers are usually coupled to HPLC. This enables the separation of complex peptide mixtures based on their molecular features before ionization and transfer into the mass spectrometer [33]. Ion signals corresponding to the peptides gathered from the mass spectrometers cannot be inferred as the absolute abundance of the protein species in a given sample for several reasons. First, the ionization efficiency can be immensely different for different types of peptides. Second, the protein purification methods in use may favor specific types of proteins at the same time, causing the loss of others. In addition, the instrument in use may have different ranges of efficiency at different sampling time points. To circumvent these issues, stable isotope techniques such as metabolic labeling or in vitro chemical labeling may be used for relative quantification of several samples [34]. The power of stable isotope-based methods is to pool various samples and measure them in the same LC-MS run. Although the labeled and unlabeled peptides cannot
Figure 3. Revealing tissue-specific FACT interacting proteins in Caenorhabditis elegans. (A) Protein extracts of wild-type N2, HMG-3::3xHA and HMG-4::3xHA strains were incubated with ChIP-grade anti-HA antibody. (B & C) Correlation matrix of CoIP-MS plotted as a heatmap, showing the Pearson correlations between control samples and anti-HA CoIP-MS experiments using HMG-3::3xHA or HMG-4::3xHA strains. (D & E) Volcano plots showing specific protein interactions of HMG-3::HA and HMG-4::3xHA based on pull-down experiments of three biological replicates. HMG-3 and HMG-4 interaction partners are shown in blue. HMG-3 and HMG-4 are shown in red. Stringency cutoffs (hyperbolic curves) are drawn with a 0.01 false discovery rate, as indicated on the volcano plot. (F & G) Tissue enrichment analysis of proteins interacting with HMG-3 and HMG-4 using WormBase WS279 (q < 0.1). (H) Venn diagram showing the overlapping interactors of HMG-3 and HMG-4. CoIP-MS: Co-immunoprecipitation with mass spectrometry. Venn diagram generated using InteractiVenn [23].
be distinguished based on their chemical composition and chromatographic behavior, MS can differentiate them based on their mass difference. However, using stable isotope labeling methods is labor-intensive, time-consuming and costly when applied in model organisms. As an alternative to label-based methods, different LFQ methods can be used. For example, simple spectral counting, such as protein abundance index [35] and extracted ion chromatography-based methods by incorporating peptide ion intensities to their chromatographic profiles [36]. These approaches can add an extra dimension to the quantification of the peptides may make extracted ion chromatography-based methods be superior to spectral counting. The advancement of label-free methods has continued and implementation of the LFQ algorithm in MaxQuant software further increased accuracy [18]. A previous study comparing metabolic labeling and label-free approaches for interaction proteomics in a mouse cell line revealed that MaxLFQ achieved quantification properties similar to those observed with SILAC, although low LFQ intensities resulted in reduced accuracy [20]. Nevertheless, for interaction proteomics, the specific interactors of a given protein are detected if their enrichment levels are significant, which makes LFQ analysis the method of choice for interaction proteomics in *C. elegans* in the authors’ study.

**Future perspective**
The LFQ analysis of HMG-3 and HMG-4 CoIP-MS in the current study as well as of MRG-1 in the accompanying study [21] delivered highly reproducible results. We confirmed predicted interactions, such as with SPT-16 [12–14], in a highly robust manner. Moreover, all 11 CoIP-MS procedures for MRG-1 showed a very strong correlation based on Pearson correlations in the accompanying study [21]. Overall, our protocol enables an efficient and robust examination of protein interactions in *C. elegans* based on CoIP-MS. For future applications, *C. elegans* provides the potential to reveal tissue-specific protein interactions, particularly when epitope-tagged proteins are expressed in confined tissue lineages. In addition, tissue-specific labeling techniques such as in vivo biotinylation [37] can be applied, and interactions can be validated via microscopy-based approaches.

**Executive summary**

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<td>To elucidate the protein–protein interactions of chromatin-regulating factors in <em>Caenorhabditis elegans</em>, the authors established a highly robust co-immunoprecipitation protocol for subsequent mass spectrometry analysis.</td>
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<td>The authors used co-immunoprecipitation in combination with single-pot, solid-phase enhanced sample preparation followed by mass spectrometry and label-free quantification.</td>
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<td>The combination with single-pot, solid-phase enhanced sample preparation provides highly robust and reproducible co-immunoprecipitation with mass spectrometry for <em>C. elegans</em> protein lysates.</td>
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<td>As a proof of concept, co-immunoprecipitation with mass spectrometry with single-pot, solid-phase enhanced sample preparation was performed for FACT subunit proteins.</td>
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<td>The FACT subunits HMG-3 and HMG-4 were tagged with a 3xHA epitope tag using CRISPR/Cas9 in <em>C. elegans</em>.</td>
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<th>Discussion &amp; conclusion</th>
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<td>Known and novel protein–protein interactions of HMG-3 and HMG-4 were identified and will be characterized to better understand how FACT regulates chromatin in <em>C. elegans</em>.</td>
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**Supplementary data**
To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2021-0074

**Author contributions**
G Baytek conceptualized the study, performed experiments, analyzed data and wrote the manuscript. O Popp helped with experiments and advised. P Mertins advised. B Tursun helped in conceptualizing the manuscript, advised and supported manuscript writing.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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