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A peptide affinity reagent for isolating an intact and catalytically active multi-protein complex from mammalian cells

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ABSTRACT

We have developed an approach for directly isolating an intact multi-protein chromatin remodeling complex from mammalian cell extracts using synthetic peptide affinity reagent **4**. FOG1(1–15), a short peptide sequence known to target subunits of the nucleosome remodeling and deacetylase (NuRD) complex, was joined via a 35-atom hydrophilic linker to the StreptagII peptide. Loading this peptide onto Strep-tactin beads enabled capture of the intact NuRD complex from MEL cell nuclear extract. Gentle biotin elution yielded the desired intact complex free of significant contaminants and in a form that was catalytically competent in a nucleosome remodeling assay. The efficiency of **4** in isolating the NuRD complex was comparable to other reported methods utilising recombinantly produced GST–FOG1(1–45).

1. Introduction

To analyse the structure and function of multi-protein complexes they must first be obtained in their native form and in sufficient quantities for biophysical and biochemical analysis.[1–3] Expression of individual subunits and in vitro reconstitution of the protein complex is often undertaken but can be difficult for multi-protein complexes with many subunits.[4,5] An alternative approach is to isolate the endogenous complex directly from cell extracts using protein-derived affinity baits.[6,7] Although this typically yields smaller quantities of protein, sufficient material can be obtained for conducting preliminary analysis on the composition, structure and functional activity of the complex.[8–10]

We are interested in using synthetic molecules to isolate intact proteins and protein complexes from cell extracts. In a typical approach, peptides and small molecules are immobilized onto biocompatible solid supports through covalent attachment or using the biotin–streptavidin interaction and used in pulldown-LC–MS/MS experiments to identify their cellular targets.[11,12] In such systems, elution of captured proteins requires harsh, denaturing conditions and the structural and functional information contained within the captured protein complex is lost. We sought to develop an approach whereby short peptide or small molecule baits could be combined with affinity tags that enable gentle elution of the captured proteins. In developing such an approach we focused on isolating the multi-subunit nucleosome remodeling and deacetylase (NuRD) complex.

The NuRD complex consists of at least 10 protein components: RBBP7, RBBP4, HDAC1, HDAC2, MTA1, MTA2, GATAD2A, GATAD2B, MBD2 or 3 and CHD4[13] (Fig. 1A). The NuRD complex is unique as a chromatin remodeler because it combines two catalytic activities—ATP-dependent chromatin remodeling and lysine deacetylation. Through these two activities it is able to convert chromatin between open, poised and closed states[14,15] to regulate gene expression. In previous work, the NuRD complex was purified in a single step from murine erythroleukemia (MEL) cell nuclear extract by affinity chromatography using GST-fusions of the first 45 residues of the transcriptional co-regulator Friend of GATA 1 (GST–FOG1(1–45))[16] and later using the first 12 residues of the FOG2 (GST–FOG2(1–12)).[17] Recently a crystal structure of the NuRD component RBBP4 bound to FOG1(1–15) was reported[18] and this, along with additional biochemical experiments, revealed an Arg-Arg-Lys-Gln motif to be central to the FOG1–RBBP4 interaction (Fig. 1B). We decided to develop synthetic molecules based on this 15-residue domain from FOG1 as baits to capture the NuRD complex from MEL cell extracts.

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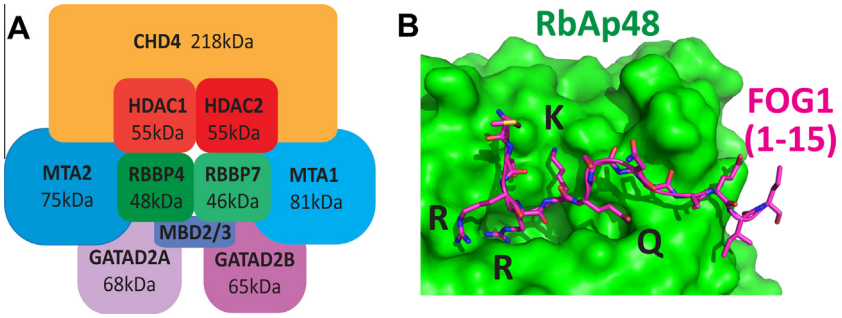

Figure 1. (A) Schematic of the nucleosome remodeling and deacetylase (NuRD) complex showing the well established subunits within the complex. (B) Crystal structure of FOG1(1–15) bound to RBBP4 (pdb 2XU7). The key RRKQ motif is shown.

2. Results and discussion

We first sought to confirm that FOG1(1–15) could indeed recruit the NuRD complex from MEL nuclear extract. We obtained FOG1(1–15) biotinylated at the C-terminus via the side chain of an additional lysine residue (bFOG1(1–15), **1**). In three parallel experiments, peptide **1** was loaded onto streptavidin beads and then incubated with a nuclear extract prepared from cultured MEL cells. Each set of beads was then washed with a different salt concentration, then the bound proteins were eluted by heat denaturation and the eluates for each experiment were analysed by SDS PAGE. Figure 2 shows the presence of many protein bands in the eluate from beads washed with 150 mM NaCl. However, when the beads were washed with higher salt concentrations (300 mM NaCl and 500 mM NaCl), fewer protein bands were observed in the eluates by SDS–PAGE. To confirm the presence of NuRD proteins, the gel lane corresponding to the eluate of beads washed with 300 mM NaCl was cut it into 10 pieces, each was individually digested with trypsin and the extracted peptides were analysed by LC–MS/MS. The NuRD components RBBP4, RBBP7, HDAC1, HDAC2, MTA1, MTA2, GATAD2A, GATAD2B, MBD3, MBD2 and CHD4 were all identified with 1–50 significant peptides and Mascot scores >500 (see Supporting information), demonstrating that **1** can recruit the entire NuRD complex. The data also show that a significant amount of non-specific binding takes place under these conditions.

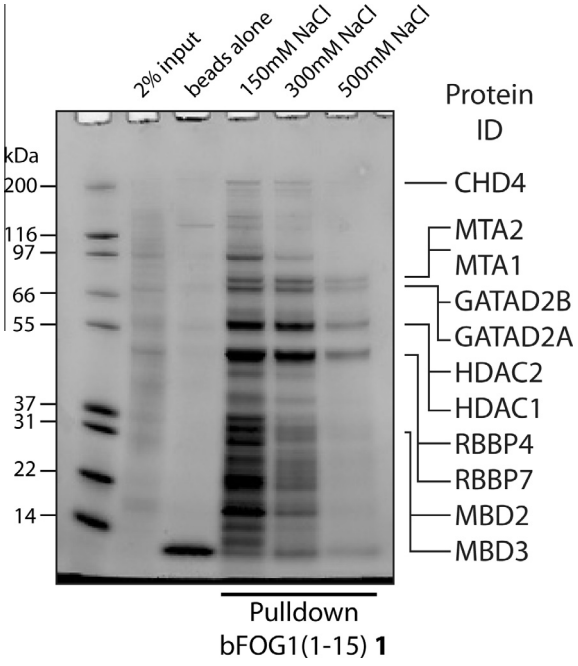

Figure 2. SDS PAGE analysis of eluates from protein pulldowns from MEL cell nuclear extract using **1** (bFOG1(1–15)) and different concentrations of salt during bead washes. Gel stained with Sypro-Ruby.

To facilitate gentle elution of the captured NuRD complex we next chose to investigate FOG1(1–15) joined to the StreptagII peptide affinity tag. This peptide tag can be loaded onto commercially available Streptactin beads and gently eluted using biotin or desthiobiotin.[19] A relative comparison of peptide tags for affinity purification indicated that the StreptagII–Streptactin system gives the best compromise between highly specific capture (e.g., relative to 6xHis-NiNTA), and cost of the affinity resin (e.g., relative to FLAG and HA antibody resins).[20] Therefore we decided to pursue this approach.

Initially we synthesised FOG1(1–15) with the eight-residue StreptagII peptide at the C-terminus (**2**, Fig. 3), loaded it onto Streptactin beads and attempted to pulldown the NuRD complex from MEL cell nuclear extracts. We attempted to elute **2**, along with any captured proteins, by three treatments with 10 mM biotin. However, no NuRD subunits were observed by SDS–PAGE in the elutions (Fig. 4A). We reasoned that upon binding to the Streptactin beads, peptide **2** might position the NuRD recruiting FOG1 domain in an awkward orientation that renders it unable to bind to RBBP4 or other NuRD components. We therefore prepared a second peptide in which the FOG1 domain and StreptagII peptide were separated by an aminohexanoic acid linker (**3**, Fig. 3) and repeated the pulldown from MEL cell nuclear extract (Fig. 4A). Once again, no NuRD proteins were observed by SDS–PAGE. Next, we decided to introduce a much longer linker between the StreptagII and FOG1(1–15). We also decided to incorporate fluorescein into the molecule so we could visually monitor peptide attachment to, and elution from, the beads.

The new peptide (**4**) was assembled entirely on solid phase according to Figure 3. After preparing the 8-residue StreptagII peptide, the bifunctional amine 4,7,10-trioxa-1,13-tridecanediamine was incorporated at the N-terminus via a urea linkage. Introduction of this linker was conducted at −20 °C to minimise hydantoin formation[21,22] which could prematurely cap the growing peptide chain. A lysine residue modified at its side chain Nε with carboxyfluorescein was then incorporated, followed by attachment of a second molecule of 4,7,10-trioxa-1,13-tridecanediamine through a urea linkage. The FOG1 peptide was then assembled to produce peptide **4** which had a 35-atom spacer between the StreptagII peptide and FOG1(1–15). This synthetic strategy gave the desired peptide in 13% purified yield and could quickly provide hundreds of milligrams that could conveniently be stored as a solid or in frozen aliquots.

Peptide **4** was then immobilized on Streptactin beads and incubated with MEL cell nuclear extract. The beads were washed in 500 mM NaCl and elution with 10 mM biotin was performed.

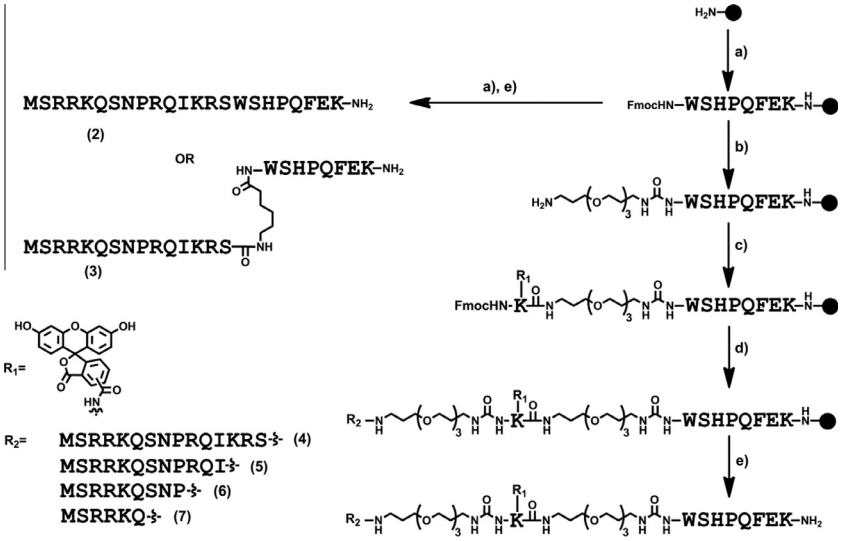

Figure 3. Synthesis of FOG1-StreptagII peptides **2–7**. (a) SPPS; (b) (i) 20% piperidine/DMF, (ii) CDI, DMF, −20 °C, (iii) 4,7,10-trioxa-1,13-tridecanediamine, −20 °C; (c) (i) Fmoc-Lys(Mtt)-OH, HBTU, DIPEA, (ii) 5% TFA/DCM, (iii) 5(6)-carboxyfluorescein, HBTU, DIPEA; (d) (i) 20% piperidine/DMF, (ii) CDI, DMF, −20 °C, (iii) 4,7,10-trioxa-1, 13-tridecanediamine, −20 °C, (iv) SPPS. (e) TFA/TIPS/H_2O/EDT/thioanisole (90/2.5/2.5/2.5/2.5).

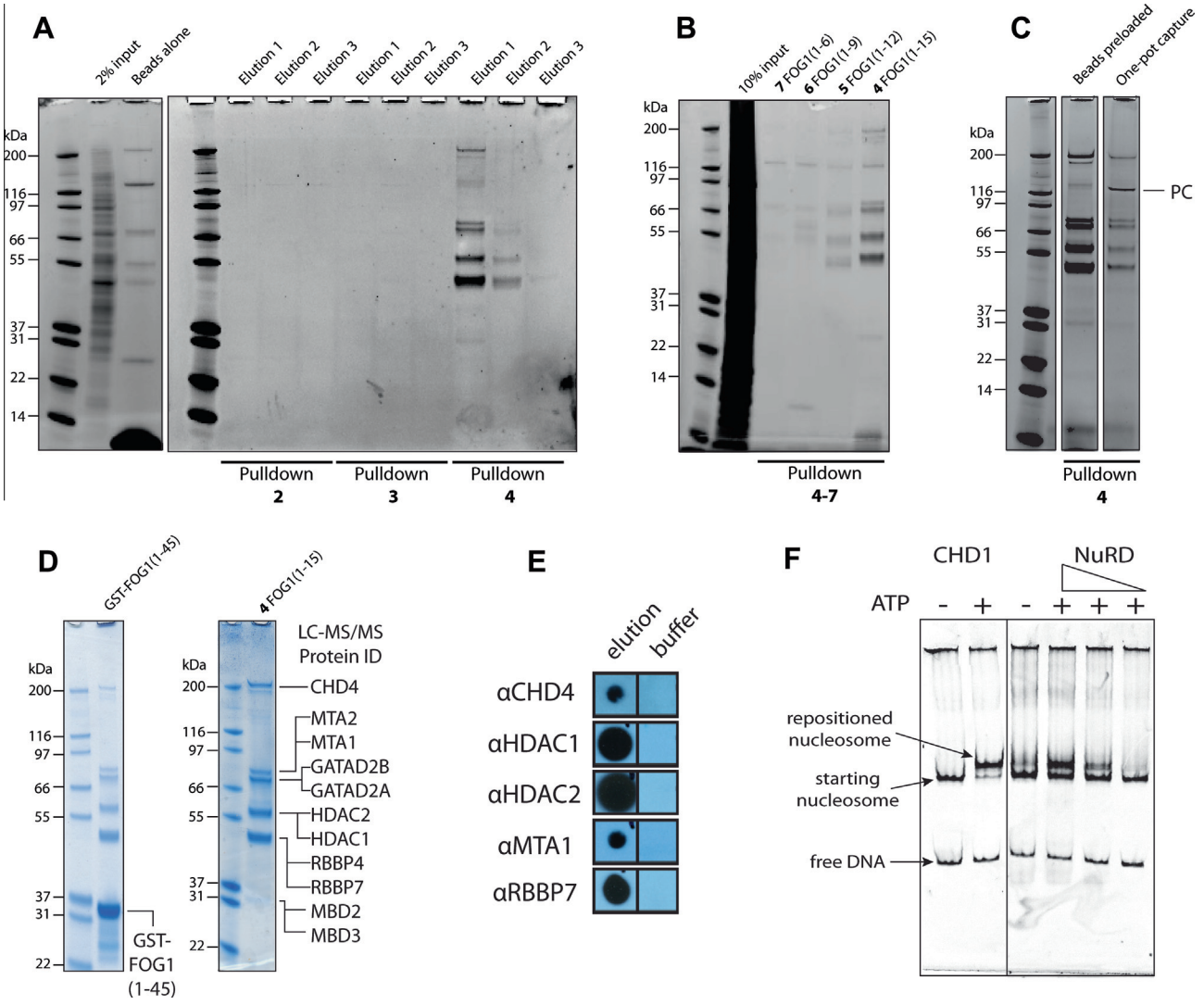

Figure 4. SDS PAGE analysis of pulldowns from MEL cell nuclear extract. (A) Using FOG1(1–15)-StreptagII peptides (**2**–**4**). (B) Using peptides **4**–**7**. (C) Comparison of NuRD capture with **4** using preloading or direct 'one-pot' incubation strategies. PC = pyruvate carboxylase. (D) Comparison of pulldowns using GST–FOG1(1–45) (left) and **4** (right). (E) Dot blot analysis of eluted proteins captured using peptide **4** or buffer. (F) Chromatin remodeling activity of eluted NuRD compared to the known remodeler CHD1.

Several clear bands corresponding to NuRD complex components were now observed by SDS PAGE in the eluted fractions (Fig. 4A). In contrast to **1**, peptide **4** was able to strongly retain NuRD proteins even in high salt conditions suggesting that the long linker may allow for optimal binding or increased avidity.

We next sought to determine whether the entire FOG1(1–15) motif was required to effectively pull-down the NuRD complex from MEL nuclear extracts. Analysis of the FOG1–RBBP4 crystal structure and previous mutagenesis data indicated that the RRKQ motif was the critical determinant of binding. We therefore expected that shorter peptides retaining this motif might still be able to pull down the complex. Three additional peptides (**5**–**7**) were prepared (Fig. 3), each with three residues successively removed from the C-terminus of the FOG1(1–15) domain.

Figure 4B shows pulldowns conducted with each of these peptides; neither **6** nor **7** were able to pull down the NuRD complex. Peptide **5** maintained some ability to pull-down NuRD, however, it was far less effective than the entire FOG1(1–15) sequence in **4**. These results suggest that truncating the FOG1(1–15) below 15 residues severely impedes its ability to bind to the endogenous NuRD complex. It is not clear why this is the case given that the RRKQ motif is preserved. Additional interactions by the three C-terminal residues may be important.

Our experiments thus far had utilised **4** that had been loaded onto Streptactin beads prior to incubation with MEL cell extract. Biotinylated small molecule affinity baits are often directly incubated with cells or cell extracts and then captured, along with their bound proteins, by incubation with streptavidin beads.[23,24] We decided to test the efficiency of this latter approach for isolating the NuRD complex using **4**. Figure 4C demonstrates that the yield of NuRD is much higher using our initial approach of preloading **4** onto the Streptactin beads prior to incubation with the cell extract. The yield of NuRD complex was much less when **4** was incubated directly with MEL cell extract and then captured using Streptactin beads. We observed an additional band by SDS–PAGE when using the direct incubation approach. LC–MS/MS determined that this protein was pyruvate carboxylase, an endogenously biotinylated protein able to bind with high affinity to the Streptactin beads.

We expressed GST–FOG1(1–45), which has previously been used to isolate the NuRD complex from MEL cells[16], and assessed its ability to pull down NuRD compared to peptide **4**. Figure 4D shows that the band pattern observed from pulldowns with GST–FOG1(1–45) was exactly the same as with peptide **4** and demonstrates that peptide reagent **4** performed as well as GST–FOG1(1–45) in pulling down the NuRD complex from MEL cell extract. Avoiding the use of GST is beneficial because of its tendency to dimerise which could complicate downstream biochemical and structural investigations. Although GST can be cleaved, it would be better not to add a protease to such a large complex with significant regions of predicted disorder that might also be susceptible to proteolysis.

To confirm that the observed bands were indeed NuRD proteins, the gel bands were excised, digested and analysed by LC–MS/MS. In all cases, the expected NuRD proteins were identified as the top protein hits (see Supporting information). A dot blot analysis was also conducted on the eluent from peptide **4** using antibodies against CHD4, HDAC1, HDAC2, MTA1 and RBBP7 (Fig. 4E). A positive result was obtained for all of the antibodies providing further evidence that the proteins observed by SDS–PAGE were indeed NuRD proteins.

Finally, we tested the ability of the NuRD complex purified using peptide **4** to reposition nucleosomes in vitro. CHD1, a known chromatin remodeler[25] was used as a positive control and could, in the presence of ATP, reposition a nucleosome in which the recombinant histone octamer is bound to a 194-bp DNA fragment. When the purified NuRD complex was incubated with the nucleosome in the presence of ATP, the appearance of a new band indicated that the histone octamer was repositioned on the DNA template (Fig. 4F) in a similar manner to CHD1. This observation indicated that the purified NuRD complex was catalytically active.

3. Conclusion

In summary, we have developed synthetic molecules capable of isolating the intact and catalytically active NuRD complex from mammalian cells. By joining FOG1(1–15) to the StreptagII peptide via a long, 35-atom linker we generated peptide **4** that was as efficient at isolating the NuRD complex as GST–FOG1(1–45) and avoided potential problems such as GST dimerisation or off-target cleavage during proteolytic GST removal. The affinity reagents described herein can be prepared in useful quantities, incorporate additional features such as fluorescent tags, can be stored for long periods at low temperature, and can be applied to any specific peptide sequence or small molecule that is known to target a specific protein or protein complex.

4. Experimental

4.1. General

bFOG1(1–15) (**1**, MSRRKQSNPRQIKRSK(biotin)) was purchased from ChinaPeptides. Fmoc-amino acids and resins were obtained from Merck (Melbourne, Australia). Dimethylformamide (DMF), trifluoroacetic acid (TFA), *N*,*N*-diisopropylethylamine (DIPEA) and piperidine were peptide grade and from Auspep (Parkville, Australia). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) was from IRIS Biotech (Germany). All other chemicals were purchased from Sigma Aldrich. Cell culture media, additives, resins, gels and enzymes were purchased from Life technologies. Preparative rpHPLC was performed on a Phenomenex C18 column (100 Å, 250 × 20 mm). Analytical rpHPLC was performed on a Phenomenex Luna 5 μm C18 column. Peptides were purified using gradient mixtures of water/0.1% TFA and 90% acetonitrile/0.1% TFA. Purity was checked under different gradients to confirm single products. Molecular weights were determined using a Voyager DE-STR MALDI mass spectrometer.

4.2. Peptide synthesis

Peptides were assembled by manual solid phase peptide synthesis (SPPS) in Teflon syringes fitted with a frit (Torviq). Peptides were synthesised on 0.1 mmol scale on Rink Amide MBHA resin (Novabiochem, 0.59 mmol/g).

4.2.1. Fmoc deprotections

The peptide resin was treated twice with 20% piperidine in DMF for 3 min. The resin was then washed with DMF five times.

4.2.2. Coupling

0.5 mmol of Fmoc-amino acid was dissolved in 1 mL of 0.5 M HBTU. 87 μL of DIPEA (0.5 mmol) was added and the mixture vortexed for 20 s and then added to the peptide resin. Couplings proceeded for 15 min at room temperature. After coupling the liquid was ejected from the syringe and capping solution added immediately.

4.2.3. Capping

55 μL acetic anhydride (0.5 mmol) was dissolved in 1 mL DMF and 87 μL of DIPEA (0.5 mmol) was added. The mixture was mixed vigorously for 20 s and then added to the peptide resin. Capping

proceeded at room temperature for 10 min after which the liquid was ejected and the resin washed twice with DMF.

4.2.4. Cleavage

Peptide resins were washed with DMF and then DCM and transferred to a scintillation vial with a stirrer bar. The resin was then treated with 90% TFA, 2.5% triisopropylsilane, 2.5% H_2O, 2.5% ethanedithiol, and 2.5% thioanisole for 2 h at room temperature. Peptides were subsequently isolated by evaporation of the cleavage mixture and ether precipitation.

4.2.5. HPLC

Peptides were purified on GBC HPLC system using a Phenomenex Jupiter C18 column (10 μm, 300 Å, 250 mm × 15 mm) linear gradient of 0–70% B over 35 min at 3 mL/min. Fractions were analysed by MALDI-MS, analysed by rpHPLC to ensure >95% purity and lyophilized.

4.3. Strep-tagged peptides (4–7)

The StreptagII peptide was assembled using standard procedures. Then the N-terminal amino group was activated with a pre-cooled (−20 °C) solution of carbonyldiimidazole (1 mmol, 10 equiv) in 2 mL DMF for 1 h at −20 °C by placing the reaction vessel in a freezer. Following washing with pre-cooled (−20 °C) solution of DMF (3 × 5 mL), a pre-cooled (−20 °C) 1:1 mixture of DMF and 4,7,10-trioxa-1,13-tridecanediamine (4 mL) was incubated with the resin for 1 h at −20 °C. Fmoc-Lys(Mtt)-OH was subsequently coupled to the N-terminus and the Mtt group was removed from the side chain epsilon amino group by 5 × 3 min treatments with 3% TFA in DCM with 3% TIPS. 5(6)-carboxyfluorescein was coupled on to the lysine side chain using HBTU/DIPEA activation and reacting with the resin overnight. Then the Fmoc group was removed and a second 4,7,10-trioxa-1,13-tridecanediamine linker was installed using CDI activation protocol above. Finally, the FOG1 peptides were assembled using standard SPPS methodology.

4.4. Cell culture

Murine erythroleukemia (MEL) cells were cultured in DMEM medium (+glucose, +glutamine, +pyruvate) supplemented with 5% FBS and 1% penicillin/streptomycin. 10–20 mL seed cultures were maintained and for large scale grow-ups a 1 mL seed culture was added to 250 mL fresh medium and grown at 37 °C, 5% CO_2 to a density of ~1×10^6 cells/mL (viability >85%). Cells were harvested by centrifugation at 2000 rpm for 5 min to yield ~1 g (wet weight) cells/L culture. Cells were washed twice with PBS, then swollen in hypotonic solution (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9) for 20 min and frozen in liquid nitrogen and stored at −80 °C until use.

4.5. Nuclear extract preparation

Frozen, swollen cells were thawed at 37 °C for 10 min. IGEPAL was added to a final concentration of 0.6% (v/v) and cells were incubated for 10 min to lyse the cell membrane. The mixture was centrifuged for 5 min at 2000 rpm to pellet nuclei and the cytoplasmic supernatant was discarded. The pellet was gently washed once with hypotonic solution (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9, 0.6% IGEPAL) and centrifuged again at 2000 rpm. Next, buffer A (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, Complete protease inhibitors, pH 7.4) was added to the pellet (3 mL/g cells) and the mixture was sonicated on ice (step-tip, 10 × 1 s bursts with 10 s recovery, three times total) to give a milky white solution. The mixture was centrifuged at 13000 rpm for 10 min at 4 °C. The clear nuclear extract was then used immediately.

4.6. Affinity resin preparation

Streptavidin or Streptactin beads 50 μL (300 nmol/mL, 15 nmol) were washed three times with water, and then three times with buffer A (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, Complete protease inhibitors, pH 7.4). Peptides (50 nmol, dissolved in 50 μL buffer A) were added to the beads and incubated at 4 °C for at least 2 h. The beads were then washed three times with buffer A and used immediately.

4.7. Pulldown

The nuclear extract was incubated with the peptide affinity resin overnight at 4 °C. The next morning the beads were separated from the nuclear extract and washed three times with high salt buffer A (50 mM Tris, 500 mM NaCl, 1% Triton X-100, 1 mM DTT, Complete protease inhibitors, pH 7.4). To Streptavidin beads in 50 μL buffer was added 10 μL gel loading dye (LDS) and the resulting sample was heated for 10 min at 90 °C and then analysed by SDS PAGE. To washed Streptactin beads was added 100 μL of elution buffer (10 mM biotin, 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, Complete protease inhibitors, pH 8.4); the mixture incubated at 4 °C for 30 min and the eluate separated from the beads. The elution protocol was conducted a total of three times to ensure all peptide/captured protein was released and individual elutions were analysed by SDS PAGE.

4.8. Mass spectrometry

MALDI: Peptide samples were analysed on a Voyager DE-STR MALDI mass spectrometer in reflectron or linear mode. α-cyanohydrocinammic acid (CHCA) matrix (1 μL, 10 mg/mL in 70% acetonitrile, 30% H_2O, 0.1% TFA) was spotted onto the plate and allowed to dry. The peptide sample (1 μL) was then spotted on top of the dried matrix and the plate dried.

LC–MS: Digested gel bands were analysed on a QSTAR Elite (ABSciex, Foster City CA) mass spectrometer coupled online to an Agilent 1100 series nano-LC system (Agilent Technologies, Santa Clara CA). Samples were resuspended in buffer A (0.1% formic acid), trapped on a Zorbax 300SB-C18 trap column (5 μm, 5 × 0.3 mm; Agilent Technologies) and then separated on a Zorbax 300SB-C18 HPLC column (3.5 μm, 150 mm × 75 μm; Agilent Technologies) using a linear gradient of 5–80% buffer B (99.9% acetonitrile, 0.1% formic acid) for 60 min at a constant flow rate of 300 nL min^{-1}. The QSTAR Elite was operated in data-dependent positive ion mode with a selected range of 350–1850 *m/z*. The three most intense ions from each MS scan (charge states 2+ to 4+) were selected for collision-induced dissociation (CID) fragmentation, with rolling collision energy enabled and a dynamic exclusion of 30 s. Raw data were viewed and scripted to Mascot generic format (.mgf) in Analyst 2.0 (ABSciex). Mascot generic format files were searched against the SwissProt/Uniprot database using an in-house Mascot server, allowing variable modifications of acetamidomethyl (Cys) and oxidation (Met), one possible missed tryptic cleavage and a peptide and protein tolerance of 0.2 Da for QSTAR Elite data.

4.9. Tryptic digestion of gel bands

Gel bands were excised with a scalpel and diced into 1 mm^3 pieces. The gel pieces were destained with a 1:1 mixture of 25 mM NH_4HCO_3 and acetonitrile (3–4 treatments for 10 min each). After destaining peptides were reduced in 50 μL of 10 mM dithiothreitol, 100 mM NH_4HCO_3 at 55 °C for 1 h and subsequently

alkylated with 50 μL of 50 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. The gel plugs were washed several times with a 1:1 mixture of 25 mM NH_4HCO_3 and acetonitrile and then dehydrated in 100% acetonitrile and dried under vacuum. 20–30 μL Trypsin (sequencing grade, Promega, 12 ng/μL) was added and the gel pieces were left to rehydrate for 1 h at 4 °C. The liquid was discarded and replaced with 50 μL 25 mM NH_4HCO_3 and the mixture was incubated overnight at 37 °C. The supernatant was removed and set aside. Another 50 μl NH_4HCO_3 was added to the gel pieces and they were sonicated for 15 min and pooled with original peptide solution. The combined peptide solution was desalted (C18) prior to LC–MS/MS.

4.10. Preparation of GST–FOG1(1–45)

FOG1(1–45) was cloned into pGEX-6P and overexpressed in the BL21(DE3) strain of *E. coli* using 1 mM IPTG induction at 18 °C for 16 h. Cell pellets were lysed by sonication (in 50 mM Tris pH 8.0, 50 mM NaCl, 0.5 mM PMSF, 0.1% β-mercaptoethanol, 0.1 mg/mL lysozyme, 10 μg/mL DNase I) and clarified by centrifugation. 25 mL of clarified solution was bound to 240 μL glutathione Sepharose beads at 4 °C for 1 h. The beads were washed several times (50 mM Tris, 500 mM NaCl, 1% Triton X-100, 1 mM DTT, Complete protease inhibitors, pH 7.4) and used directly in pull-down experiments.

4.11. Dot blot analysis

An elution from a peptide-**4** pulldown or a buffer control were spotted onto activated PVDF membrane and the membrane blocked using 4% skim milk powder in PBS-T. The membrane was then washed three times for 5 min with PBS-T before incubation with primary antibody overnight. The primary antibodies used were rabbit αCHD4, mouse αHDAC1, mouse αHDAC2, rabbit αMTA1 and rabbit αRbAp46. αCHD4 and αRbAp46 were used in a 1:1000 dilution in PBS-T with 5% BSA and αHDAC1, αHDAC2 and αMTA1 were used in a 1:2500 dilution in PBS-T with 5% skim milk powder. After overnight incubation membranes were washed with PBS-T three times for 5 min before incubation with appropriate secondary antibody in 1:3000 dilution in PBS-T with 5% skim milk powder for 1 h. Membranes were again washed before being visualized using Western Lightning™ Illumination kit (PerkinElmer LAS, Inc.) with a variety of exposure times.

4.12. ATP-driven remodeling reactions

Production and purification of recombinant *Saccharomyces cerevisiae* CHD1(ΔC1305), nucleosome assembly and the ATP-driven sliding assays were performed essentially as described in.[25,26] Briefly, nucleosomes were assembled using standard salt-gradient dialysis methods using recombinant *Xenopus laevis* histones and PCR-generated Cy3-labelled DNA fragments containing the 147 bp 601 nucleosome positioning sequence flanked by 47 bp DNA on one side. The sliding reactions contained 50 mM Tris pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, 0.5 pmol of nucleosome, and 1 mM ATP (where indicated). Reactions were started by the addition of remodeling enzyme, CHD1 (3.75 pmol) or NuRD (1.5, 0.5, 0.17 μL), and were incubated at 37 °C for 1 h. The reactions were stopped by placing on ice and the addition of 500 ng sheared salmon sperm DNA and sucrose to 5% w/v. Products were resolved on a 0.25 × TBE 6% native polyacrylamide gel run for 50 min at 200 V at 4 °C. Gels were imaged on an FLA-9000 fluorescence scanner.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.01.023.

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