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Quantitative analysis of cohesin complex stoichiometry and SMC3 modification-dependent protein interactions

Chen Ding[#], **Yehua Li**[#], **Beom-Jun Kim**[#], **Anna Malovannaya**, **Sung Yun Jung**, **Yi Wang**, and **Jun Qin**[*]

Center for Molecular Discovery, Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, USA.

Abstract

Cohesin is a protein complex that plays an essential role in pairing replicated sister chromatids during cell division [1-3]. The vertebrate cohesin complex consists of four core components including structure maintenance of chromosomes proteins SMC1 and SMC3, RAD21 and SA2/SA1. Extensive research suggests that cohesin traps the sister chromatids by a V-shaped SMC1/SMC3 heterodimer bound to the RAD21 protein [4] that closes the ring. Accordingly, the single 'ring' model proposes that two sister chromatids are trapped in a single ring that is composed of one molecule each of the 4 subunits. However, evidence also exists for alternative models. The hand-cuff model suggests that each sister chromatid is trapped individually by two rings that are joined through the shared SA1/SA2 subunit. We report here the determination of cohesin subunit stoichiometry of endogenous cohesin complex by quantitative mass spectrometry. Using qConCAT-based isotope labeling, we show that the cohesin core complex contains equimolar of the 4 core components, suggesting that each cohesin ring is closed by one SA1/SA2 molecule. Furthermore, we applied this strategy to quantify post-translational modification-dependent cohesin interactions. We demonstrate that quantitative mass spectrometry is a powerful tool for measuring stoichiometry of endogenous protein core complex.

Keywords

cohesin complex; qConCAT; mass spectrometry; stoichiometry; immunoprecipitation

Introduction

Cohesin is a protein complex that functions in fundamental biological process – sister chromatid cohesion - that pairs replicated sister chromatids during cell division [1-3]. The vertebrate cohesin complex consists of four core components including structure maintenance of chromosomes proteins SMC1 and SMC3, RAD21 and SA2/SA1 (also known as stromal antigens STAG2 and STAG1) [1, 2, 5]. SMC1 binds SMC3 to form a V-shape molecule that is closed by binding to the RAD21 protein [4]. Accordingly, the widely

[*]To whom correspondence should be addressed. jqin@bcm.edu.
[#]These authors contributed equally.

accepted 'single ring' model of the cohesin-DNA interaction proposes that cohesin forms a ring which traps the two sister chromatids together and this model is supported by the experimental evidence that two sister mini-chromosomes are entrapped by single cohesin ring [6]. However, alternative models have been proposed where each cohesin complex binding to each sister chromatid forms oligomers (snap model) or cohesin complex oligomerizes to form filament (bracelet model) [7]. Among them, one of the compelling models is the handcuff model, where two cohesin complexes, each with one encircled chromatid, are joined through the interaction with SA1/SA2 [8]. This model is supported by the experimental evidence that SMC1, SMC3, and Rad21 can coimmunoprecipitate themselves while SA1/2 cannot bind to itself. Because the major difference between the single ring model and handcuff model is the oligomerization of the cohesin rings, knowing the stoichiometry of the cohesin complex can determine which of these models is likely to be correct. Several cohesin binding proteins also play important roles in regulating cohesin establishment and maintenance. PDS5 and PDS5A and PDS5B, two homologues of PDS5 in metazoans, are also essential for maintenance of cohesion in G2/M phase [9-11]. Another essential cohesin regulator is SORORIN (also known as CDCA5), which was identified as a substrate of APC/C^{cdh1}, an ubiquitin ligase active from late mitosis to G1 phase. SORORIN is necessary for cohesin establishment and maintenance [12], and interaction between SORORIN and cohesin is dependent on SMC3 acetylation and DNA replication [13, 14]. Conversely, WAPL play a role in removing cohesin from chromatin in mitosis and also might affect cohesin binding to DNA in interphase [15, 16].

In addition, cohesin is regulated by post-translational modifications (PTMs). Acetylation of SMC3 in yeast and human by the establishment of cohesion acetyltransferase ESCO1, a human homolog of Eco1, is required for the establishment of cohesion in S-phase [17-19]. In response to DNA damage, multiple cohesin subunits, including SMC1, SMC3, and Mcd1, the budding yeast RAD21 homolog, are phosphorylated by the checkpoint kinase ATM/ATR and Chk1[20-23]. However, the molecular mechanism by which these PTMs regulate cohesion is still not clear. One possibility is that the PTMs may alter protein-protein interactions within the cohesin complex.

Mass spectrometry (MS) has emerged as an indispensable analytical tool for protein analysis in biochemistry and proteomics. It has been widely used for the identification of proteins and the sites of post-translational modifications (PTMs) by taking advantage of its qualitative properties. Meanwhile, the demand for quantitative MS has increased, and a number of techniques are being actively developed for the relative and absolute quantification of proteins using isotope labeled standards [24]. The synthetic isotope labeling of standard reference peptides and metabolic isotope labeling in cell culture are two common strategies for comparative quantification of changes in relative abundance of the same peptide in two different samples. Stable isotope labeling with amino acids in cell culture (SILAC) is the most commonly used method for metabolic labeling [25]. In this method, two groups of cells are grown in cell culture media that are identical except that one contains light isotope labeled amino acids and the other contains heavy isotope counterparts labeled with the combinations of ^{13}C or ^{15}N. These two cell cultures are then mixed and processed, and the amounts of a given protein under these two culturing conditions can be distinguished and quantified on the MS spectrum by the mass difference and the respective

intensities of heavy and light peptide peaks. While SILAC has been widely used in quantification of the same protein under different conditions, it is not suitable for quantification of different proteins under any conditions. An alternative labeling method for relative quantification across different peptides has been developed[26]. In this approach, a synthetic gene encoding an artificial concatenation of tryptic peptides from a set of different proteins (a qConCAT protein) is cloned into an *E.coli* expression vector and expressed in a medium containing stable heavy isotope labeled lysine and arginine [26]. A known amount of the qConCAT protein is then co-digested with the analyte proteins; and the ratio of the digested proteins is determined by comparing the relative intensities of the analyte peptides that have been normalized to their corresponding isotope-labeled qConCAT peptides. This technique has been successfully applied to quantify multiple proteins expressed during muscle development [27]. Later, a modified qConCAT method was used to measure stoichiometry of a biochemically purified transducin protein complex [28]. Here, we report the development of a qConCAT method for measuring the stoichiometry of the human cohesin protein complex, as well as a quantitative study of the PTM-dependent protein-protein interactions. We investigated parameters that affect the cohesin qConCAT proteins and optimize the selection of qConCAT peptides, and devised general strategies for the measurement of endogenous protein complex stoichiometry. Our data support the single "ring" model of the cohesin complex. We also applied the qConCAT approach to quantify changes in protein-protein interactions associated with the cohesin complex as a function of SMC3 post-translational modifications. Our work reveals a possible role of SMC3 acetylation in modulating protein interaction within cohesin complex.

Materials and Methods

The qConCAT proteins

The qConCAT cDNA was reverse translated from amino acid sequence of the selected qConCAT tryptic peptides and chemically synthesized (Gene 2.0, CA). The cDNAs were cloned into pGEX4T-1 vector.

Isotope labeling of the qConCAT proteins

The GST-qConCAT plasmids were transformed into bacterial strain BL21 cells for protein expression. Fresh *E. coli* BL21 culture was inoculated in 5 ml of heavy SILAC DMEM medium (Invitrogen, CA; $^{13}C_6$ Arginine and $^{13}C_6$ Lysine, without glutamine) and grown at 37°C for 16 hours.IPTG (0.4 mM) was added to the bacterial culture when it reached a cell density of ~0.5 OD_{600nm}/ml to induce the qConCAT protein expression at 37°C for 3 hours. The expression and identity of the recombinant protein was verified by Coomassie Brilliant Blue (CBB) staining and mass spectrometry.

GST-qConCAT protein purification

The BL21 cells were collected and suspended in NETN buffer [150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.8), 1% NP-40, with protease inhibitors] and the lyzed on ice by sonication. The lysate was centrifuged at 60,000 rpm for 10 min and the supernatant was collected to purify GST-qConCAT recombinant protein using GSH beads. The purified

GST-qConCAT proteins were eluted by elution buffer [10 mM glutathione, 50 mM Tris-HCl (pH8.0), 5% glycerol] and stored at -20°C before use.

Immunoprecipitation

MCF7 or HeLa cells were lysed in the HEPES-based lysis buffer [50 mM HEPES (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20, 10% glycerol and a protease inhibitor mixture] or NETN. Cohesin complex from the chromatin-bound fraction was solubilized by sonication and total cohesin was immunoprecipitated with antibody cross-linked protein A Sepharose beads. Flag-SMC3-WT, -K105A/K106A, -S1067A, -S1083A and -K105A/K106A/S1083A stable inducible cell lines were generated using the Flp-In T-REX™ system (Invitrogen, CA) in 293T cells as described previously [17, 23]. Exogenous SMC3 expression was induced by doxycycline at concentration of 1 μg/ml and immunoprecipitated with Flag M2-agarose (Sigma).

Tryptic co-digestion of protein complex with the qConCAT protein

For in-solution digest, thecohesin complex was immunoprecipitated and eluted by 30% acetonitrile solution with 1% formic acid. The eluate was dried in a Savant SpeedVac vacuum centrifuge, re-dissolved in 50 μl of 50mM NH_4HCO_3, and spiked with an aliquot (100ng) of the purified heavy qConCAT protein. The solution was then digested with 1 μg of trypsin at 37°C overnight.

Quantitative MS

The purified GST-qConCAT peptides were analyzed by a LTQ-Velos-Oribitrap mass spectrometer with a 65 min acetonitrile gradient. The raw data were collected in a data dependent mode in which the precursors were scanned by the Orbitrap (target resolution 100K), fragmented by collision induced dissociation (CID) and analyzed by LTQ-Velos. The experimental m/z and retention time of qConCAT peptides were recorded qConCAT for full MS quantification. We used the peak area calculation function in Xcalibur Qual Browser (v 1.0.042) to calculate the AUC for each qConCAT peptide; the mass tolerance was set as 10 ppm. Each mass spectrometer used here was equipped with a nanospray ion source, an HPLC and an autosampler system (Thermo Fisher).

Monitoring proteolysis and dynamic range of qConCAT peptide

qConCAT protein was digested by trypsin at 37°C for different times. For measurements of the qConCAT peptide response-curves, the qConCAT protein was digested overnight (~ 16 hours). The peptide solution was then titrated and quantified by MS as described in the text.

Results

Optimize and validate qConCAT standards

The mass spectrometric behaviors of the tryptic peptides used for the qConCAT protein determine the accuracy of the qConCAT quantification. The criteria for choosing the peptides are as follows: (1) can be are completely and accurately digested by trypsin (no miscleaved cutting sites) within short time; (2) strong MS response; (3) absence of

methionine, cysteine and tryptophan, which can be oxidized and complicate the quantification, and (4) an optimal length (10-20 amino acids) and appropriate hydrophobicity. Furthermore, it is always desirable to measure multiple peptides for each analyte protein to assure consistency in quantification. We designed the qConCAT protein as a tandem fusion of two peptides from each of the cohesin subunits SMC1, SMC3, RAD21, and peptides that allow distinction of SA1 and SA2, and PDS5A and PDS5B proteins, along with the pan-SA1/2 and pan-PDS5 peptides, to investigate the potential isoform-specific cohesin complex combinations. We synthesized the DNA sequence encoding this qConCAT protein, and sub-cloned it into a pGEX4T-1 vector with an N-terminal GST epitope tag to facilitate qConCAT protein solubilization and purification (Figure 1). We found that the heavy DMEM medium used for SILAC for mammalian cells can be used to culture *E.coli* and express the recombinant isotope-labeled qConCAT protein. This alternative labeling media is of considerably low cost than heavy isotope chemical labeling. Considering that bacteria generally uses glutamine to synthesize arginine [29], which causes the incorporation of light isotope-labeled arginine, we therefore utilized glutamine-free DMEM medium for qConCAT expression. Over 99% of the qConCAT proteins grown in the heavy isotope SILAC DMEM incorporated labeled lysine and arginine (Table 1 and Figure 2A).

Next, we used in-solution digestion to characterize the mass spectrometric response of the qConCAT peptides. We reasoned that, comparing to in-gel digestion, in-solution digestion has higher accuracy and reliability by alleviating differences in selection of gel range and peptide extraction. The measurements of the recovered peptides and their response curves for the peptides in the qConCAT protein indicate that they possess the right attributes for the qConCAT-based quantification, namely, the complete digestion and good linear responses with similar response slopes of large values.

We then test the robustness of this method for the determination of stoichiometry. We mixed equal amount of heavy and light labeled qConCAT proteins and measured the ratio of H/L stoichiometry using SMC1 amount as 1. As expected, and the H/L stoichiometry for all the components is 1 +/- 0.1, suggesting that this approach has the accuracy of ~10% variation (Figure 2B). Figure 1 summarizes the optimal workflow of choosing tryptic peptides for the qConCAT standards.

Determine the stoichiometry of the cohesin complex—Since the interaction between core subunits of a complex is best preserved, we first used a SMC1 antibody to immunoprecipitate the cohesin complex. As shown in Figure 3A, we obtained an approximately 1:1 molar ratio between SMC1 and SMC3, consistent with the fact that SMC1 and SMC3 form a stable heterodimer. However, in the same experiment, the stoichiometry of all core cohesin components SMC1:SMC3:RAD21:SA1/2 was determined to be 1:1:0.15:0.06 – a value that seems to be at odds with any feasible cohesin structure. There are at least three possible explanations for this experimental observation: (1) antibody displacement, where binding of an antibody to one antigen changes the conformation of the complex and leads to dissociation of the other components, (2) differential dissociation during purification, where varying degrees of dissociation are observed for different interactions based on their affinity and arrangement, *e.g.* the dissociation between SMC1

and SMC3 is less than that of RAD21 with SMC1/3 dimer, or (3) complex heterogeneity, where multiple isoforms of cohesin complex with different composition and/or stoichiometry contribute to the observed molar ratio of the population of immunoprecipitated cohesin complexes.

To circumvent these problems, we tested whether the immunoprecipitation of a peripheral component of the cohesin complex could help avoiding antibody displacement of the core components. PDS5A is considered a peripheral component because it can be washed away from cohesin without disrupting the four-subunit cohesin core. We reasoned that while the antibody displacement can also occur in this case, it is much more likely to cause the loss of the whole core cohesin complex, rather than any one of its subunits preferentially. The remaining core complex will then maintain its internal stoichiometry in the experimental results, even if its unit ratio to the peripheral antigen is distorted. We isolated PDS5A together with cohesin using an antibody against PDS5A immobilized on the protein A beads. The target PDS5A protein with the associated cohesin complex was then eluted, digested in-solution together with the qConCAT standard, and quantified with a LTQ Velos Orbitrap mass spectrometer (Figure 3B). MS measurement reveals that the core cohesin complex, which binds PDS5A, exhibits a 1:1:1:1 ratio for all core components (SMC1, SMC3, RAD21 and SA1/SA2), consistent with the single ‘ring’ model proposed by Gruber *et al* [6, 30]. Because our qConCAT peptides contained pan-SA peptides as well as SA1- and SA2-specific sequences, we were able to distinguish SA1- and SA2-specific cohesin isoforms and show that 80% of PDS5A-interacting cohesins contain SA2, whereas 20% contain SA1. This is a clear example of the protein complex heterogeneity. The similar design for PDS5A/B quantitation also allowed us to conclude that the anti-PDS5A antibody is indeed PDS5A specific, and that PDS5A- and PDS5B-containing cohesins are also highly likely to be exclusive of each other, since no PDS5B detected in PDS5A-IP complex (Figure 3B). The ratio of WAPL to the core cohesin complex also measured at 1:1. Since WAPL is known to bind PDS5A[16] , there are two possible interpretations of the 1:1 WAPL:cohesin ratio. One interpretation is that WAPL only binds the whole PDS5A cohesin complex stoichiometrically; the other is that WAPL is partially displaced by the PDS5A antibody, and the 1:1 ratio to the core cohesin complex is simply coincidental.

Quantify PTM-dependent protein-protein interactions in the cohesin network —Next, we sought to apply the qConCAT method to the quantification of the PTM-dependent interactions of the SMC3 protein complexes. Acetylation and phosphorylation of SMC3 have been shown to play important roles in cohesin function [17, 23, 31]. Here, we tested whether the changes in function of the SMC3 mutants can be attributed to the modulation of protein interactions within the cohesin network.

We made Tet-inducible Flag-SMC3-WT, -K105A/K106A, -S1067A, -S1083A and -K105A/K106A/S1083A stable cell lines in 293T cells. We then isolated the different Flag-SMC3 proteins using anti-Flag M2 beads, digested the immunoprecipitated proteins with the heavy isotope-labeled qConCAT protein, and quantified the cohesin stoichiometry on the Orbitrap mass spectrometer (Figure 4). The mutations of K105 and K106 residues on the SMC3 preclude acetylation by ESCO1 and cause cohesion defects in human cells. We found that this SMC3 mutant has no impact on the interaction between core cohesin components, and

confirmed the acetylation-dependent decrease of core cohesin interaction with the peripheral components PDS5A/B and WAPL, which is consistent with previous reports [32, 33]. In contrast, our results suggest that phosphorylation of SMC3 at S1067 and S1083 have the opposite effect to the acetylation, namely, they increase the interaction of SMC3 with PDS5A/B and WAPL as this interaction decreases by phosphorylation-deficient mutation. The effect of the S1083A mutation on the association with PDS5 and WAPL proteins appears to be less pronounced than that of the S1067A mutant. The phosphorylation at S1083 also strengthens the binding between SMC3/SMC1/RAD21 trimer and SA1/2. Quite interestingly, a peripheral component SORORIN (CDCA5) was enriched by 10 fold in the immunoprecipitates of SMC3-K105A/K106A acetylation mutant, revealing a possible role of SMC3 acetylation in regulating the SORORIN association within the complex. Recently, it was found that more SORORIN binds to cohesin complex when these acetylation sites are mutated [14] and our data are consistent with these results. To confirm this observation that SORORIN prefers to associate in the cohesin complex in which SMC3 acetylation is defective, we tried to dissociate SORORIN from the cohesin complex using two buffers of either stringent detergent (0.5% Tween-20, 10% glycerol) or milder one (0.5% NP-40). As shown in Figure 5, even by stringent buffer wash, SORORIN can't be easily depleted from the SMC3-K105A/K106A cohesin complex in comparison to the SMC3 wild type or S1083 mutant complex. This data suggests that SORORIN not only accumulates, but also binds much more tightly in SMC3-K105A/K106A cohesin complex. Furthermore, we conclude that acetylation of SMC3 is dominant over S1083 phosphorylation, because the effect of the combined K105A/K106A/S1083A mutation on protein association within the cohesin complex mirrors that of the acetylation mutant.

Discussion

Since different peptides display different response curves in mass spectrometry, the conventional metabolic (SILAC) isotope-labeling methods have been largely restricted to measurements of changes in the same peptide across different experimental conditions. The AQUA (absolute quantification) technique and its variations were developed to quantify the absolute amount of a given peptide [34-36], paving the way for quantitative comparison of different proteins. However, the synthetic isotope labeling of peptides can be costly, and, more importantly, the absolute amount of peptide may not accurately reflect the amount of corresponding protein, because efficiencies of protein digestion and peptide extraction are unlikely to be 100% and equivalent among different peptides.

The invention of the qConCAT method not only offers a cost-efficient alternative to obtain heavy isotope labeled standards, but also minimizes experimental variations, because all the steps after electrophoresis are performed simultaneously for both analytes and the qConCAT reference protein. One of the inherent advantages of qConCAT is that the molar ratio between each peptide can be set to 1:1 by design and used to normalize different mass spectrometric responses of peptides, allowing relative quantification of different proteins.

We report the development of the qConCAT-based method to measure stoichiometry of the endogenous human protein complexes. Importantly, we investigate the general principles and suggest a practical approach for the optimal peptide choice of the qConCAT reference

peptides that can be easily applied to any protein complex of interest. By investigating immunoprecipitations of different cohesin complex components, we show that cautions should be taken in the interpretation of the measured molar ratios, particularly when the immunoprecipitation is carried out from the core components. The complications in deriving the stoichiometry of a protein complex from the qConCAT data for an IP sample stem from experimental artifacts such as displacements caused by antibody binding, which likely affects the IPs of core components, and the differential dissociation of subunits under the strain of the purification procedure. In addition, heterogeneity of the protein complexes, their relative fractional distribution within the cell, and antibody preferences will weigh into the observed ratios. Accurate stoichiometry measurement of the components may be more readily obtained by immunoprecipitation of a peripheral component that binds to the core complex, although the stoichiometry between the peripheral component and the core might be distorted. We applied this logic in investigation of the stoichiometry of the cohesin core complex and obtained a 1:1:1:1 ratio for the core components of SMC1, SMC3, RAD21, and SA1/2. These results provide direct analytical evidence to support the ‘ring’ model for cohesin. We also successfully utilized our qConCAT procedure to measure the PTM-dependent protein interactions within the cohesin network.

Measurement of cohesin complex stoichiometry using qConCAT

The accuracy of the qConCAT method relies primarily on the choice of the qConCAT peptides. By carefully examining the MS responses and trypsin digestion kinetics for the cohesin peptides, we found that the ideal qConCAT peptides can be best selected based on early appearance in tryptic digests. This peptide behavior correlates well with the demonstration of good linear response and sensitivity in the MS over a large concentration range. The low variation and high experimental reproducibility of the qConCATv3-based measurements validates this strategy for choosing the qConCAT candidates.

The stoichiometry of the cohesin complex is a fundamental consideration for the understanding the cohesin-DNA interactions. The widely accepted single ‘ring’ model predicts that two DNA fibers are trapped in a single ring-like cohesin complex where the stoichiometry of the core components SMC1:SMC3:Rad21:SA1/2 is 1:1:1:1. Several alternative models exist that predict higher-order interactions of the cohesins [7]. In particular, Zhang *et al.* proposed a ‘handcuff’ model where two SMC1/SMC3/RAD21 rings, each with one chromatid, are bridged by the SA1/2 subunit. They showed a possibility of SMC1-to-SMC1 and SMC3-to-SMC3 association that is SA1/SA2-dependent [8]. This model predicts that a cohesin complex stoichiometry with a 2:2:2:1 ratio of SMC1:SMC3:Rad21:SA1/2. The exact ratio of the cohesin core complex components was not explicitly measured and described before our study.

Although it is considered most convenient to isolate the core complex using antibodies against the core components, we found that such isolation procedure may disturb the stoichiometric ratio between the main subunits, likely by antibody displacement. Since antibody displacement is an inherent property of the experiment, it should be noted that exhaustive repetition of the same experiments offers no further insights into stoichiometry of the complex. Furthermore, we also showed that the use of multiple antibodies to different

components is unlikely to definitively resolve these artifacts. Thus, while isolation of multiple core components may be the shortest route to determine protein complex composition, it may not the best optimized strategy for inferring stoichiometry. Rather, the solution is to isolate the core complex through a peripheral component that interacts with the core complex, but with much weaker affinity. It this situation, any disturbance, whether by antibody, or by the strain of the procedure, will lead to the total loss of the core components, not differential loss of certain components as is often the case when core components of a stable complex are precipitated. With a properly designed and characterized qConCAT method, we successfully measured the stoichiometry of the endogenous cohesin core complex to 1:1:1:1 for the SMC1:SMC3:RAD21:SA1/2 protein, thereby providing strong support for the 'single ring' model of the cohesin complex. While this manuscript was in preparation, Hotzmann and colleagues reported mass spectrometric-based determination of cohesin core complex stoichiometry[37]. Using label-free and absolute quantification, these authors derived same 1:1:1:1 stoichiometry for the cohesin core complex, and a relative abundance of STAG1 and STAG2 containing cohesin to be 6-8% to 92-94% respectively.

Traditional methods for absolute protein quantification requires large amount of sample, which makes the measurement of an endogenous immuno-purified complex difficult. The qConCAT method, as described here, provides sufficient sensitivity to measure the stoichiometry of the endogenous protein complexes at relatively low cost and effort. The amount of protein needed is often below the detection limit (20 ng) of Coomassie Blue staining. This opens a door for measuring the stoichiometry of any protein complexes that can be conveniently isolated by immunoprecipitation.

SMC3 PTM-dependent protein interactions within the cohesin network

A logical extension of this qConCAT application is the measurement of differences in the PTM-dependent protein interactions, a knowledge that may provide deeper insights into the understanding of how post-translational modifications execute the cellular changes. SMC3, one component of the cohesin complex has various PTMs including acetylation, phosphorylation, methylation and each PTM has an important role in cohesin functions [17-19, 23, 33, 38]. Using modification-deficient mutants of SMC3 and qConCATv3 protein, we investigated the PTM-dependent changes in the cohesin interaction network. An intriguing observation we found is the acetylation-dependent binding of SORORIN. SORORIN is required for sister chromatid cohesion in higher eukaryote [5, 39] and SMC3 acetylation is necessary for SORORIN function [13, 14].Our data thus reveals a possible mechanistic role of SMC3 acetylation and supports recent finding that SORORIN binding to cohesin complex is regulated by acetylation.

The qConCAT method has low intrinsic variation of ~10% and good linear responses, and thus is better suited than quantitative Western Blotting to determine the small changes. By the qConCAT method, we found that the majority of tested PTMs of the SMC3 protein do not impact the interaction of the core cohesin complex, but instead change the interactions with the accessory components PDS5A/B and WAPL. In addition to the dramatic change of SORORIN, another most appreciable change, (which is still less than 2 fold and not easy to quantify by Western blotting) is acetylation-dependent dissociation of PDS5A/B and

WAPL. Again, it supports previous finding that acetylated SMC3 decreases its binding to PDS5 and WAPL [32, 33]. Because of their inhibitory role in cohesin establishment, the dissociation of the PDS5/WAPL module upon SMC3 acetylation provides a feasible explanation as to how acetylation promotes sister chromatid cohesion.

The SMC3 phosphorylation is known to regulate DNA damage response [23]. In this study, we found that PDS5A/B, WAPL, and SA1/2 binding were decreased in SMC3 phosphorylation-deficient mutants. These three proteins are suggested to form a module of cohesin that has anti-establishment activity in the process of cohesion [40]. In addition, the phosphorylation of the S1083 on SMC3 is detectable in S-phase (data not shown) in the absence of exogenous DNA damage. Given that S1083 is located proximal to the ATPase domain of SMC3, which executes the cycles of cohesin opening and binding to the DNA, the finding of the increased SA/PDS5/WAPL binding with core cohesin suggests that not only acetylation, but also phosphorylation of SMC3 might play a role in cohesin establishment in cycling cells.

Taken together, we optimized the qConCAT method and measured the stoichiometry of the endogenous cohesin complex by qConCAT. The result suggests that sister chromatid cohesion is mediated by a single cohesin ring. In addition, change of protein interaction caused by SMC3 post-translational modification can be detected by this improved qConCAT method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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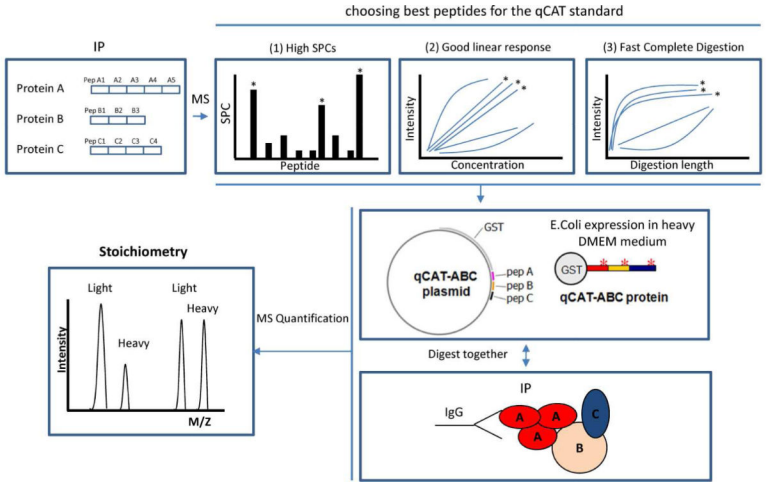

Figure 1. Optimal workflow for selection of tryptic peptides for the qConCAT standard

To select best peptides for the qConCAT standard, the immunoprecipitated (IP) cohesin complexes were digested and measured by MS. In addition to the accepted physical and chemical characters, an ideal qConCAT peptide should have (1) high spectral counts (SPCs) in an IP, (2) a linear response curve with the best sensitivity to concentration changes, and (3) show shortest length of time for the complete tryptic digestion in-solution. Two qConCAT peptides are selected for each protein complex subunit. These are fused in tandem to design a qConCAT standard in silico and reverse-translated. The qConCAT gene was chemically synthesized, sub-cloned in an expression vector, and expressed in *E.coli* cultured in glutamine-free DMEM medium containing heavy lysine and arginine. The immunoprecipitated protein complex and heavy isotope labeled qConCAT are combined in same tube for in-solution digestion. Each pair of analyte peptide and qConCAT peptide is quantified using mass spectrometry.

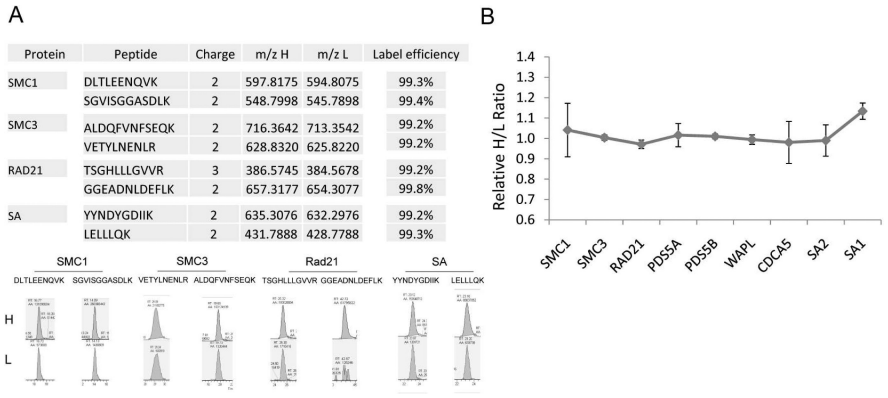

Figure 2. Optimization and characters of the peptides selected for the cohesin complex qConCAT standard

(A) Labeling efficiency of heavy isotope- qConCAT standard is more than 99%. (B) Validation of stoichiometry measurement of light and heavy labeled qConCAT protein. Equal amount of H and L qConCAT proteins were mixed and the relative ratio of the H/L values were measured by comparing their corresponding peak area of heavy and light isotope labeled qConCAT peptides. All H/L ratios were normalized to the SMC1. The error range represents measurements for the two qConCAT peptides for each of the cohesin proteins.

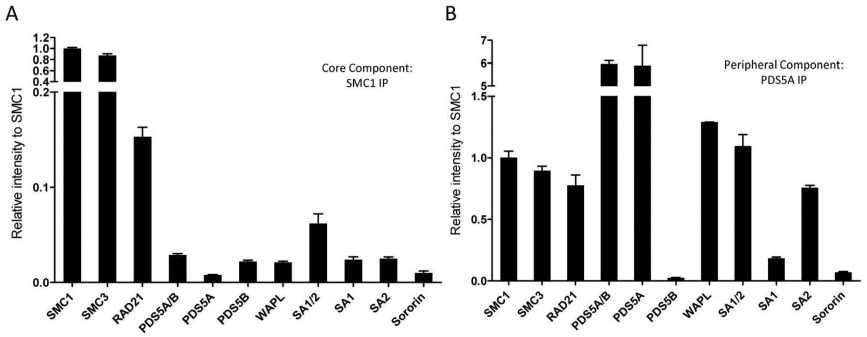

Figure 3. Cohesin complex stoichiometry in SMC1 and PDS5A immunoprecipitations

The cohesin complexes were immunoprecipitated from the MCF7 whole cell extracts and quantified against the qConCATv3 standard. Protein amounts in the two IPs were normalized to their respective antigens. (A) IP of the core cohesin component SMC1 shows potential displacement of RAD21 and SA1/2. (B) IP of the peripheral cohesin component PDS5A, where stoichiometry of the core cohesin complex (SMC1, SMC3, RAD21, and SA1/2) appears equimolar. Furthermore, PDS5A cohesins do not contain PDS5B, and ~20% of cohesin complexes contain SA1, whereas the other ~80% contains SA2.

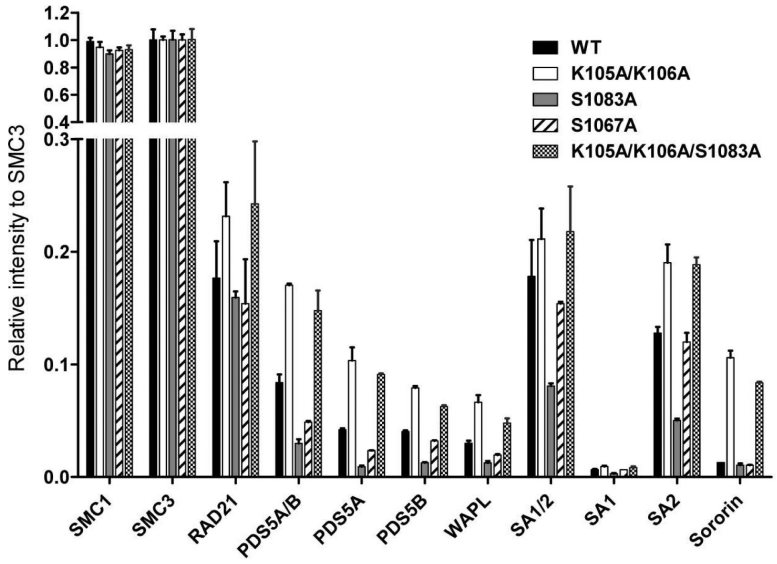

Figure 4. qConCAT quantification reveals changes in protein associations of the cohesin complexes containing PTM-deficient mutants of SMC3

Flag-SMC3-WT and PTM-deficient SMC3 mutant were immunoprecipitated with anti-Flag M2 Sepharose and quantified against the qConCATstandard. The amount of each complex component was normalized to that of SMC3.

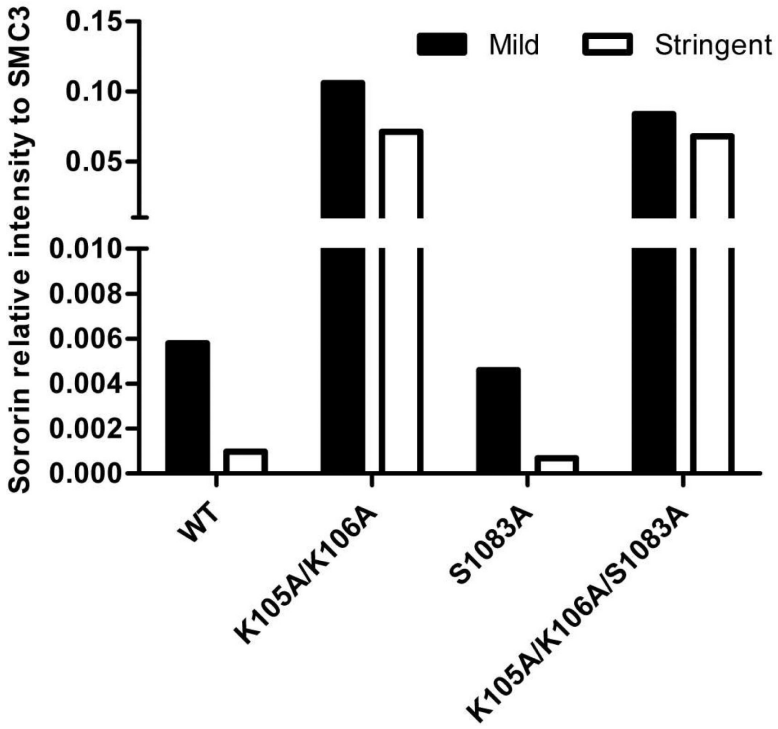

Figure 5. Comparison of relative amount of SORORIN with two washing conditions from wild type or PTM defective SMC3 immunoprecipitations

Flag-SMC3-WT and PTM-deficient SMC3 mutants were immunoprecipitated and washed by two buffers with different detergent contents to dissociate SORORIN from cohesion immunoprecipitates. The relative amount of SORORIN protein level is normalized to that of SMC3.

Table 1

Selected qConCAT peptides of cohesin complex.

Protein	Peptides	Sequence
SMC1	SMC1-1	DLTLEENQVK
	SMC1-2	SGVISGGASDLK
SMC3	SMC3-1	ALDQFVNFSEQK
	SMC3-2	VETYLNENLR
RAD21	RAD21-1	TSGHLLLGVVR
	RAD21-2	GGEADNLDEFLK
PDS5A	PDS5A-1	SIEGTADDEEEGVSPDTAIR
	PDS5A-2	HDVIVTIITAAK
PDS5B	PDS5B-1	SIDGTADDEDEGVPTDQAIR
	PDS5B-2	FTQVLEDDEK
WAPL	WAPL-1	ISHVVVEDTVVSDK
	WAPL-2	LLELEQDASSAK
SA1	SA1-1	NSLVTGGEDDR
	SA1-2	SQLIDEFVDR
SA2	SA2-1	GVVTAEMFR
	SA2-2	LELFTSR
pan-SA	SA1/2-1	YYNDYGDIIK
	SA1/2-2	LELLLQK
pan-PDS5	PDS5A/B-1	DLTEYLK
	PDS5A/B-2	ALNEMWK
SORORIN	SORORIN-1	LETLGSASTSTPGR
	SORORIN-2	THSVPATPTSTPVPNPEAESSSK