Novel Essential DNA Repair Proteins Nse1 and Nse2 Are Subunits of the Fission Yeast Smc5-Smc6 Complex*

Received for publication, August 11, 2003, and in revised form, August 29, 2003
Published, JBC Papers in Press, September 8, 2003, DOI 10.1074/jbc.M308828200

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The structural maintenance of chromosomes (SMC) family of proteins play essential roles in genomic stability. SMC heterodimers are required for sister-chromatid cohesion (Cohesin: Smc1 & Smc3), chromatin condensation (Condensin: Smc2 & Smc4), and DNA repair (Smc5 & Smc6). The SMC heterodimers do not function alone and must associate with essential non-SMC subunits. To gain further insight into the essential and DNA repair roles of the Smc5–6 complex, we have purified fission yeast Smc5 and identified by mass spectrometry the co-precipitating proteins, Nse1 and Nse2. We show that both Nse1 and Nse2 interact with Smc5 in vivo, as part of the Smc5–6 complex. Nse1 and Nse2 are essential proteins and conserved from yeast to man. Loss of Nse1 and Nse2 function leads to strikingly similar terminal phenotypes to those observed for Smc5–6 inactivation. In addition, cells expressing hypomorphic alleles of Nse1 and Nse2 are, like Smc5–6 mutants, hypersensitive to DNA damage. Epistasis analysis suggests that like Smc5–6, Nse1, and Nse2 function together with Rhp51 in the homologous recombination repair of DNA double strand breaks. The results of this study strongly suggest that Nse1 and Nse2 are novel non-SMC subunits of the fission yeast Smc5–6 DNA repair complex.

Maintenance of genomic integrity is crucial for cell viability and suppression of cancer priming genetic alterations. The genome cycle requires large amounts of DNA to be packaged and structurally manipulated, during replication, mitosis and repair. A superfamily of proteins called the structural maintenance of chromosomes (SMC) family play essential roles in all of these processes (reviewed in Refs. 1 and 2). Eukaryotes contain 6 SMC proteins, called SMC1–6. All SMC family proteins share a similar architecture, in that they contain N- and C-terminal Walker A and Walker B motifs, respectively, separated by an extensive region that forms a coiled-coil structure. These SMC proteins form specific heterodimers producing three distinct SMC complexes involved in different aspects of DNA metabolism. SMC1 and SMC3 form the basis of the cohesin complex that tethers sister chromatids together following passage of the replication fork. SMC2 and SMC4 are required for chromosome condensation during mitosis, and SMC5 and SMC6 are required for DNA repair as well as an enigmatic essential function (reviewed in Refs. 1 and 2).

The SMC heterodimers do not function alone but require interaction with essential proteins called non-SMC subunits. Fission yeast cohesin (Smc1–3) requires the non-SMC subunits, Rad21 and psc3 (3). Rad21 is cleaved by separase (Esp1) prior to anaphase, abolishing sister chromatid cohesion and allowing chromosomes to segregate (3). An intriguing model has recently been proposed in which Smc1–3 encircle sister chromatids, with the circle held closed by interaction of Rad21 (Scc1) with the head groups of the asymmetric Smc1–3 heterodimer (4). Cleavage of Rad21 (Scc1) by separase (Esp1) results in breakage of the Smc1–3 circle and loss of cohesion between sisters.

Condensin requires at least three non-SMC subunits; Cnd1, Cnd2, and Cnd3 to compact chromosomes during mitosis (5). The non-SMC subunit requirements of the fission yeast Smc5–6 complex are currently poorly defined. Studies in fission yeast showed that the Smc5–6 complex appears to consist of six additional as yet unidentified non-SMC subunits (6). Determining the function of the Smc5–6 complex would be greatly assisted by identification and study of its non-SMC subunits.

Mutations have been isolated in SMC and non-SMC components of cohesin and condensin that yield DNA damage-sensitive phenotypes. For example, hypomorphic Rad21 mutants display sensitivity to radiation (7). A recent study shows that cohesin may play direct roles in DNA repair at the damage site, rather than just maintaining chromosome cohesion (8). In mammalian cells, cohesin is specifically recruited to the sites of laser-induced DNA damage (8). Notably, the fission yeast condensin mutant cnd2-I maintains cell viability but renders cells sensitive to UV irradiation and replication arrest (9). How condensin effects DNA repair is unknown.

The Smc5–6 complex is often labeled as a DNA repair complex. Indeed, Smc6 (Rad18) was first identified in screens for DNA damage-sensitive mutants (6, 10, 11). It is certainly involved in DNA repair but Smc5–6 is essential even in the absence of extrinsic DNA damage. Based on the essential nature of Smc5–6 and the fact that mutants of cohesin and condensin yield DNA damage sensitivity, it is likely that Smc5–6 has a genome "housekeeping" role. Support for such a role comes from the fact that in fission yeast, Smc6 (Rad18) mutations are synthetic lethal with topoisomerase II mutations (11).

Recently, physical and genetic interactions were detected between Rad60 and the Smc5–6 proteins (12, 13). Like
Smc5–6, Rad60 is essential and plays a role in DNA repair. The similarity between the phenotypes of Smc5–6 and Rad60 mutants suggests a co-dependent function of Rad60 and Smc5–6; however, the precise nature of the relationship needs further study.

The identification and characterization of non-SMC subunits of cohesin and condensin greatly facilitated functional analysis of these complexes. Therefore, to gain insight into the function of the Smc5–6 complex and potentially Rad60, we purified Smc5-TAP from fission yeast and applied multidimensional protein identification technology (MudPIT) to identify co-purifying proteins (14–16).

Here we report the identification of two non-SMC subunits of the fission yeast Smc5–6 complex. Recently, the identification of a budding yeast non-SMC component of the Smc5–6 complex called NSE1 was described (17). We found very weak homology between NSE1 and one of the proteins we identified. Therefore, to maintain a unifying nomenclature we refer to this fission yeast protein as non-SMC element Nse1 and the other as Nse2.

Gel filtration analysis shows that Nse1 and Nse2 co-migrate with the characteristic high molecular weight Smc5–6 complex (6). Co-immunoprecipitation studies confirmed that Nse1 and Nse2 are indeed part of the Smc5–6 complex in vivo. Nse1 and Nse2 are both essential genes, hypomorphic mutations of which yield DNA damage-sensitive phenotypes. Epistasis analyses suggest that, like Smc5–6, Nse1 and Nse2 function in the homologous recombination repair of DNA double-strand breaks with the fission yeast Rad51 homologue, Rhp51. Together, these data strongly suggest that Nse1 and Nse2 are non-SMC subunits of the fission yeast Smc5–6 DNA repair complex.

EXPERIMENTAL PROCEDURES

General Techniques—Standard fission yeast methods and media were used in these studies (18). UV and IR sensitivity assays were performed as described (19).

Generation of Tagged, Deleted, and Mutated Genes—Nse1 and Nse2 were deleted by replacement of the entire open reading frame (start to stop codon) of each gene with the kanMX6 module as described in Bähler et al. (20) producing heterozygous diploids (20). Epitope-tagged Nse1, Nse2 and Smc5 were also generated as described in Bähler et al., 1998, using the PCR-based method to place a Myc or TAP epitope at the C terminus of each protein and mark the allele with the kanMX6 gene. The tagged proteins were confirmed as fully functional.

The nse1–1 and nse2–1 alleles were generated using PCR. Genomic DNA was isolated from yeast containing the epitope-tagged nse1-1-myc: kanMX6 and nse2-2-myc:kanMX6 alleles. The entire genome locus containing each allele was amplified by PCR using standard conditions (from start codon to 100-bp downstream of KanMX6). The amplified loci were then re-amplified in 4 parallel PCR reactions. The PCR reactions were pooled and transformed into Schizosaccharomyces pombe using the transformation protocol described in Bähler et al. (20) and transformants were selected by growth on YES media containing G418 (to select for nse1-1 and nse2-2 alleles by the transformed nse1-1-myc:kanMX6 and nse2-2-myc:kanMX6 alleles as described in Bähler et al. (20). Stable transformants were then tested for temperature sensitivity and drug sensitivity by plating the strains on YES media at 36 °C or on YES plates containing 5 mM hydroxyurea. Strains that displayed temperature and/or hydroxyurea sensitivity were transformed with an episomal plasmid containing the wild-type genomic nse1 or nse2 genes to confirm that the strain defects were rescued by and therefore, alleles were to the respective genes.

Immunoblotting and Microscopy Techniques—Immunoblotting was performed as described using extracts made from cells lysed in a bead beater (19). Briefly, cells were lysed using in buffer A (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, 5 μg/ml each of leupeptin, pepstatin, and aprotonin, and 1 mM phenylmethylsulfonyl fluoride) and resolved in 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Proteins were transferred to Immobilon membrane, blocked in 5% milk in TBS and 0.3% Tween-20, and probed with antibodies to the epitope. Nse1-myc and Nse2-myc were detected with anti-Myc antibody (9E10 at 1:5,000; Santa Cruz Biotechnology). Smc5-TAP was detected with peroxidase anti-peroxi-
FIG. 1. Nse1 and Nse2 peptides identified by mass spectrometry. A, alignment of Nse1 with homologues from budding yeast and humans. Sequences aligned are fission yeast Nse1 (Nse1, 232 amino acids; SPCC550.05), human Nse1 (HuNse1, 256 amino acids; accession no. AAH18938) and budding yeast NSE1 (NSE1, 336 amino acids). The Nse1 peptides obtained by mass spectrometry analysis are represented by bold type. Identical residues between sequences are boxed and shaded. Asterisks (*) are placed under each zinc ligand of the predicted Nse1 zinc finger. The right hand panel is a table showing the number of independent peptides and percent primary sequence coverage obtained by mass spectrometry for each protein indicated. B, sequences aligned are fission yeast Nse2 (267 amino acids; SPAC16A10.06c), human Nse2 (HuNse2, 247 amino acids; accession no. AAH32797) and budding yeast MMS21 (267 amino acids). The Nse2 peptides obtained by mass spectrometry analysis are represented by bold type. Identical residues between sequences are boxed and shaded. Asterisks (*) are placed under each zinc ligand of the predicted Nse2 zinc finger.
identifies more than 100 unique proteins, most of which are not detectable by silver staining of gels. We therefore use immunoblotting to confirm that TAP epitope-tagged proteins have been efficiently precipitated before subjecting samples to mass spectrometric analysis. We have performed many TAP purifications of nuclear and cytosolic proteins under comparable conditions, allowing us to generate a list of common background proteins (e.g. ribosomes, metabolic enzymes, and actin).

The list of proteins identified in the Smc5-TAP purification was compared with this list to exclude common background. For proteins that are unique to a particular purification, we use the number of peptides obtained, and the percentage coverage of each protein’s primary sequence as a rough indication of their relative abundance.

As expected, mass spectrometric analysis of the affinity purified Smc5-TAP sample revealed extensive peptide coverage of both Smc5 and its heterodimeric partner Smc6 (81.6 and 75.6% respectively; Fig. 1A, right panel). Interestingly, as judged by the extensive peptide coverage of each, two novel proteins were also stably co-precipitated with Smc5-TAP. Nse1 was identified by peptides covering 73% of its 232 amino acid primary sequence (Fig. 1A). The data base annotation for this gene is currently incorrect as it lacks an additional 3-prime exon. The annotated open reading frame encodes a protein of only 203 amino acids. This 203 amino acid truncated form of Nse1, unlike the 232 amino acid protein, is unable to complement the Nse1 deletion strain (data not shown). When the mass spectrometry data was again searched with full-length Nse1, we...
obtained peptide coverage of the 29 amino acids encoded by the extra exon. Importantly, the full-length Nse1 contains a RING-finger like domain (26), found in the Nse1 homologues from different species (Fig. 1A). Recently, NSE1 of budding yeast was described as a novel subunit of the SMC5–6 complex (17). We found that fission yeast Nse1 shows low homology to NSE1. Although BLAST scores are not statistically significant (expect 6e-06; Refs. 27–29). The presumptive Nse2 homologues all share the highest sequence homology across their C-terminal regions, which contain a potential zinc stabilized structure. Interestingly, hypomorphic mutations in the essential gene MMS21 result in hypersensitivity to DNA damaging agents (27–29). However, the role of MMS21 in DNA repair and its protein partners are currently unknown. In particular, MMS21 has not been described as part of the budding yeast SMC5–6 complex.

In Vivo Interaction of Nse1 and Nse2 with Smc5 and Smc6—We identified Nse1 and Nse2 as Smc5-TAP co-purifying proteins using mass spectrometry. The mass spectrometry data showed that Nse1, Nse2, and Smc6 stably co-precipitated with Smc5-TAP. To obtain independent confirmation of this observation, we epitope-tagged Nse1 and Nse2 at their genomic loci with the Myc epitope. Strains containing both Smc5-TAP and Nse1-myc or singly tagged strains were generated using standard yeast genetics. We found that Nse1-myc was readily detected in Smc5-TAP immunoprecipitates (Fig. 2A). Neither Nse1-myc nor cross-reacting bands were observed in the negative control lanes (Nse1-myc, Smc5-TAP; Fig. 2A). A comparison of the input signals for Smc5-TAP and Nse1-myc with the signals for each protein following precipitation, suggests that the interaction of Smc5-TAP with Nse1-myc is avid (Fig. 2A). Although stoichiometry cannot be judged due to the different epitope tags, the ratio between the Smc5-TAP and Nse1-myc signals before and after precipitation did not change markedly (exposures were directly comparable). Nse2-myc, like Nse1-myc, specifically associates with Smc5-TAP (Fig. 2B).

The apparent stability of the Smc5-TAP Nse1-myc/Nse1-myc interaction suggested that Nse1 and Nse2 might be subunits of the Smc5–6 DNA repair complex. Gel filtration analysis has previously established that Smc5 and Smc6 are part of a large (~1.6 MDa) complex, containing about six other unidentified proteins (6). To determine whether Nse1 and Nse2 are part of this Smc5-Smc6-based complex, we used double-tagged strains containing both Smc5-TAP and Nse1-myc or Nse2-myc. Comparison of the gel filtration profile of Smc5-TAP with those of Nse1-myc and Nse2-myc, shows that both Nse1-myc and Nse2-myc co-fractionate with Smc5-TAP (Fig. 2, C and D). Based on the above co-precipitation and gel filtration data, Nse1 and Nse2 appear to be subunits of the Smc5–6 complex in fission yeast.

Nse1 and Nse2 Are Essential Genes Encoding Nuclear Proteins—If Nse1 and Nse2 play a direct role in DNA metabolism in the Smc5–6 complex, then they should localize to the nucleus as observed for Smc5 and Smc6 (11). To determine the localization of Nse1 and Nse2, we tagged each protein at its genomic locus with a C-terminal GFP epitope. Both fusion proteins were expressed from their endogenous promoters and were fully functional. Consistent with Nse1-GFP and Nse2-GFP being resident in the Smc5–6 complex, both are predominantly nuclear proteins at all stages of the cell cycle (Fig. 3, A and B and data not shown).

Smc5 and Smc6 are essential genes in both fission and budding yeasts (6, 10, 17). If Nse1 and Nse2 are functional subunits of the Smc5–6 complex we anticipated that they should

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also be essential. Heterozygous diploids were made in which the entire open reading frame of both Nse1 and Nse2 were replaced with the kanMX cassette (20). Sporulation of the heterozygous Nse1 and Nse2 diploids produced no viable kanamycin resistant colonies (not shown). To confirm these results, we dissected tetrads and observed 2:0 segregation of viability and the kanMX phenotype (Fig. 3, C and D). Cells deleted for Nse1 and Nse2 germinated and formed microcolonies of elongated and sick cells before growth ceased (Fig. 3, C and D). This terminal phenotype matches well that observed for Smc5–6-deleted cells (6, 10). Together, these results show that, like SMC5 and SMC6, both Nse1 and Nse2 are essential for cell growth.

Characterization of Hypomorphic Alleles of Nse1 and Nse2—Hypomorphic alleles of both Nse1 and Nse2 were generated to study the DNA repair roles of these essential proteins. We made a bank of Nse1 and Nse2 mutants using a mutagenic PCR strategy (see “Experimental Procedures”) and here we present the characterization of the temperature sensitive mutants nse1-1 and nse2-1. Growth of nse1-1 and nse2-1 at permissive temperature (25 °C) was not significantly different from that of the same strains carrying wild-type nse1 or nse2 on a plasmid (Fig. 4A, left panels). However, at restrictive temperature (36 °C), the growth of nse1-1 and nse2-1 cells carrying the control plasmid was severely impaired in comparison to nse1-1 and nse2-1 cells containing the wild-type gene on a plasmid (Fig. 4A, middle panels). These results demonstrate that, as expected, the temperature-sensitive phenotypes of both nse1-1 and nse2-1 are derived from mutations within the nse1 and nse2 genes. The nse1-1 and nse2-1 cells are sensitive to UV irradiation (Fig. 4A, right panels), as are cells containing Smc6 hypomorphic alleles (10, 11). The UV sensitivity of both nse1-1 and nse2-1 cells is rescued by copies of the respective wild-type genes on an episome, showing that the UV sensitivity of these strains is derived from mutations within the nse1 and nse2 genes (Fig. 4A, right panels).

Inactivation of Smc5 and Smc6 results in cell death, with various terminal morphologies including; highly elongated cells with fragmented nuclei and small “cut” cells in which the nucleus has been bisected by the septum and elongated cells with diffuse or fragmented DNA (DAPI in lower panels).

**Fig. 4.** Characterization of hypomorphic alleles of Nse1 and Nse2. A, temperature-sensitive mutants of Nse1 and Nse2, nse1-1, and nse2-1 respectively, were transformed with a plasmid containing the wild-type gene or a vector only control. Serial dilutions (2500, 500, 100, and 20 colony forming units) of the resultant strains were spotted onto plates and grown under the indicated conditions. At 25 °C all strains grew well. At the restrictive temperature of 36 °C, the plasmid-borne wild-type copies of Nse1 and Nse2 specifically rescued the temperature sensitive phenotypes of nse1-1 and nse2-1. The strains were also exposed to UV irradiation and grown at the permissive temperature of 25 °C. Again, the plasmid-borne wild-type copies of Nse1 and Nse2 specifically rescued the UV sensitivity of nse1-1 and nse2-1. B, pictures of the temperature-sensitive phenotypes of nse1-1 and nse2-1 cells grown at restrictive temperature for 8 h. Control wild-type cells show normal nuclear morphology and cell size. However, both nse1-1 and nse2-1 cells display a number of aberrant phenotypes (white arrows) including; cut cells in which the nucleus has been bisected by the septum and elongated cells with diffuse or fragmented DNA (DAPI in lower panels).
with Nse1 and Nse2 acting as non-SMC subunits of the Smc5–6 complex, we find that inactivation of nse1-1 and nse2-1 produces strikingly similar phenotypes to those observed for Smc5–6 mutants (Fig. 4B). At restrictive temperature, the nse1-1 and nse2-1 mutants display a mixture of elongated cells with fragmented or diffuse chromatin and “cut” cells, as compared with wild-type cells under the same conditions (Fig. 4B).

In quantitative analyses, both nse1-1 and nse2-1 cells display hypersensitivity to UV irradiation (Fig. 5A). In addition to enhancing survival of UV-induced DNA damage, the Smc5–6 DNA repair complex is important for the repair of DNA double-strand breaks (DSBs) caused by ionizing radiation (10). Likewise, the nse1-1 and nse2-1 mutations result in reduced viability in response to γ-irradiation at the permissive temperature for these mutants (Fig. 5B). A major pathway for the repair of DSBs is homologous recombination (HR), in which the broken DNA ends invade an intact homologous template (e.g. sister chromatid), priming repair synthesis. The initiating step of HR is largely dependent on Rhp51 (Rad51) and therefore, mutations in genes required for HR are often epistatic to rhp51 mutants. Such epistasis with rhp51 was previously observed for hypomorphic alleles of Smc6, in response to ionizing radiation (10). We find that nse1-1 rhp51 and nse2-1 rhp51 double mutants are no more sensitive to γ-irradiation than the rhp51 single mutant, suggesting that these proteins function in the same DSB repair pathway (Fig. 5B). The sensitivity of nse1-1 and nse2-1 mutants to DNA-damaging agents and their epistasis with rhp51 provides further strong support for Nse1 and Nse2 being functional subunits of the Smc5–6 complex.

**DISCUSSION**

In this study we identify two novel proteins that interact with the Smc5–6 complex in fission yeast. Since the discovery of the multiprotein Smc5–6 complex, the non-SMC subunits have remained elusive, hampering its functional analysis (6). Nse1 and Nse2 co-precipitate with Smc5-TAP and co-migrate with the previously described Smc5–6 complex. Both Nse1 and Nse2, like Smc5–6, are essential for cell growth, and hypomorphic mutants display DNA damage hypersensitivity. In addition, Nse1 and Nse2 play a role in the repair of DNA double-strand breaks that appears to be dependent on the recombination repair protein, Rhp51. Importantly, fission yeast Smc5–6 was also shown to be involved in an Rhp51-dependent pathway for the repair of double strand breaks (6, 10). Taken together, these observations make a strong case for Nse1 and Nse2 being non-SMC subunits of the Smc5–6 DNA repair complex.

As expected for subunits of a conserved SMC complex, homologues of Nse1 and Nse2 are found in all species examined from yeast to man. Examination of the primary sequences of Nse1 and Nse2 does not give a clear indication of their functions. However, both contain what appear to be zinc finger-like structures. Nse2 contains a zinc finger-like motif that shows some similarity to the Miz1/Siz1/PIAS/ARIP3 family (30–32). Members of this family contain a MIZ-type zinc finger and play roles in transcription regulation, chromatin structure and septin conjugation to the small ubiquitin-like modifier SUMO (30–32). Nse2 lacks the potential DNA binding SAP domain of the Miz1/Siz1/PIAS/ARIP3 family, a motif found in diverse nuclear proteins involved in chromosome structure maintenance (33).

Interestingly, the essential protein of budding yeast MMS21, which we believe to be an Nse2 homologue; was identified in the Smc5–6 DNA repair complex. Both Nse1 and Nse2 interact with the Nse2 homologue, MMS21, in response to DNA damage and have elevated levels of mitotic recombination that are dependent on Rad52 and therefore, homologous recombination (27–29). The localization, protein partners and DNA repair pathways of MMS21 are currently unknown. Our studies suggest that MMS21 is likely a nuclear subunit of the budding yeast SMC5–6 complex.
Our interest in the Smc5–6 complex of fission yeast was prompted by the physical and genetic interactions that others and we observed with Rad60 (12, 13). Rad60 is a novel and essential factor required for homologous recombination repair of DNA double strand breaks (12, 13). Rad60 is also regulated by the replication checkpoint, via Cds1-dependent phosphorylation (12). The physical interaction between Rad60 and Smc5–6 is much weaker than the interaction between Nse1/Nse2 and Smc5–6 (12). However, due to the similarities in the DNA damage sensitivities and terminal phenotypes of Rad60 and Smc5–6 mutants, we suggest that the observed interaction is significant. We note that similarly weak interactions between bona fide components of the cohesin complex exist. The Psc3 non-Smc subunit of fission yeast cohesin is very loosely associated with the complex, but is nevertheless essential for its function (3). In addition, Scc2/Scc4 of budding yeast are not stoichiometric subunits of the cohesin complex, but are required for the loading of cohesin onto chromosomes (36). Perhaps Rad60 plays an analogous role in the interaction of Smc5–6 with chromosomes. The precise nature of the role that Rad60 plays in the Smc5–6 complex remains to be elucidated. Maybe of importance is the observation that Rad60 contains a C-terminal motif that is closely related to SUMO-1 (12). Given that Nse1 and Nse2 both contain zinc finger domains, predicted to be involved in ubiquitin-like protein metabolism, it will be interesting to test possible interactions between Rad60 and these motifs. In summary, we have isolated and characterized two novel non-SMC proteins that interact with fission yeast Smc5–6, study of which should help precipitate an understanding of the essential and DNA repair roles of this intriguing complex.

Acknowledgments—We thank Paul Russell, Eishi Noguchi, and Chiaki Noguchi for kind gifts of strains and reagents in support of this work. We also thank Clare McGowan and Paul Russell for valuable comments on the manuscript and members of the Scripps Cell Cycle Groups for support and encouragement.

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J. Biol. Chem. 2003, 278:45460-45467.
doi: 10.1074/jbc.M308828200 originally published online September 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308828200

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