p97 ATPase, an ATPase involved in membrane fusion, interacts with DNA unwinding factor (DUF) that functions in DNA replication

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Abstract DNA unwinding factor (DUF) unwinds duplex DNA and is supposed to function in DNA replication in *Xenopus* egg extracts. Here we report the isolation and analysis of a DUF-interacting factor. By immunoprecipitation, we found that p97 ATPase (p97) interacts with DUF in *Xenopus* egg extracts. This interaction was confirmed by the in vitro binding of purified p97 with DUF. When sperm chromatin was added to *Xenopus* egg extracts to construct nuclei active in DNA replication, p97 was incorporated into the nuclei. These data suggest that the complex of DUF and p97 may function in DNA replication.

Key words: DNA replication; DNA unwinding factor; p97 ATPase; *Xenopus* egg extract

1. Introduction

We recently reported the purification of a factor termed DNA unwinding factor (DUF) from *Xenopus* egg extracts [1]. DUF binds to and unwinds closed circular duplex DNA in the presence of eukaryotic topoisomerase I. Immunodepletion of DUF from *Xenopus* egg extracts reduces DNA replication activity in the extracts, and the re-addition of purified DUF to the DUF-depleted extracts restores replication activity. These data suggest the involvement of DUF in DNA replication. DUF consists of 140 kDa (DUF140) and 87 kDa (DUF87) subunits. Molecular cloning of both subunits revealed the following. The amino acid sequence of DUF140 shows significant homology to the sequence of yeast Cdc68p, which regulates the transcription of some genes in the G1 phase, probably by affecting chromatin structure [2,3]. DUF87 containing an HMG box in the C-terminal region shows sequence homology to mouse T160, which binds to the V(D)J recombination signal sequence [4], human SSRP1, which has high affinity for cisplatin-modified DNA [5], and *Saccharomyces cerevisiae* Pob3p, which interacts with Cdc68p [6]. Interestingly, the complex of Cdc68p and Pob3p was shown to bind to Pol1p, the catalytic subunit of DNA polymerase α [6], suggesting that the Cdc68p–Pob3p complex participates in DNA replication in budding yeast. Moreover, a recently found transcriptional factor, FACT, which facilitates transcription elongation from the chromatin template, turned out to be a complex of SSRP1 and the human homologue of yeast Cdc68/Spt16 protein [7,8]. These results suggest that DUF may function in the regulation of diverse DNA-related phenomena such as replication, transcription, and recombination.

p97 ATPase (p97) is a member of the AAA ATPase family and shares a duplicated region of 230 amino acid residues called the AAA motif. ATPases in the AAA family execute a diversity of cellular functions, such as membrane fusion, vesicle-mediated transport, and gene expression [9]. Putative homologues of p97 have been found in many eukaryotes, including Cdc48p in *S. cerevisiae* [10], and VCP (valosine-containing protein) in bovine, human, murine, and porcine [11]. p97 exists in the cytoplasm and the nuclei [12], and it is well known that p97 is involved in membrane fusion in the cytoplasm [13–15]. The function of p97 in the nucleus is not yet understood. However, Madeo et al. recently reported that Cdc48p enters the nucleus during late G1 phase [16]. This suggests that Cdc48p would play some roles in DNA replication.

In this study, in order to further characterize the biological function of DUF, we searched for DUF-binding proteins in *Xenopus* egg extracts by immunoprecipitation with anti-DUF140 antibody. p97 was precipitated with DUF from *Xenopus* egg extracts and extracts of cultured *Xenopus* somatic cells. Moreover, using a cell-free system for nuclear reconstitution in *Xenopus* egg extracts, we found that p97 was incorporated into the nuclei reconstituted from sperm chromatin, which shows a high level of DNA replication.

2. Materials and methods

2.1. Preparation of antisera and affinity purification of antibodies

The antisera against DUF140 were prepared as described before [1]. The polyclonal antibody against p97 was prepared as follows. The 3′-terminal portion of p97 cDNA (nucleotides 1758–2750) was amplified by PCR. The amplified fragments were cloned into pET-15b and expressed in BL21. Most of the expressed p97 fragments were recovered in the insoluble fraction. The inclusion bodies were solubilized with 6 M urea and a histidine-tagged p97 fragment was purified on Pro-Bond resin (Invitrogen) according to the manufacturer's protocol and further purified by electrophoresis. The gel was stained briefly with Coomassie brilliant blue, and the protein band was cut out. Expressed p97 fragments were extracted from the gel, dialyzed against phosphate-buffered saline, and used as the antigen for immunization of a rabbit.

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IgG was purified from serum using protein A-Sepharose as described [17]. The antibodies specific for DUF and p97 were affinity-purified as described [19].

2.2. Immunoprecipitation

Xenopus egg extracts were incubated with anti-DUF140 antibodies or preimmune IgG chemically crosslinked with dimethylpimelimidate to protein A-Sepharose for 60 min at 4°C. Proteins bound to the matrix were recovered by centrifugation and the resins were washed with NP-40 lysis buffer (1.0% Nonidet P-40, 0.15 M NaCl, 50 mM Tris–HCl (pH 8.0)) [17]. The bound proteins were eluted with 0.1 M glycine-HCl (pH 2.8), separated by SDS-PAGE, and detected by silver staining or Western blotting using anti-DUF140, anti-DUF87, or anti-p97 antibodies.

2.3. Peptide microsequencing

Partial amino acid sequences of the DUF-associated 100 kDa protein were determined as described [18].

2.4. Purification of DUF and p97

DUF was purified from *Xenopus* egg extract as described [1].

p97 was purified from *Xenopus* egg extract as follows. NaCl was added to a *Xenopus* egg extract to a final concentration of 0.2 M, and the extract was centrifuged at 200 000×*g* for 1.5 h. The supernatant was subjected to gel filtration on a G3000SW column equilibrated with buffer A (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.2 M NaCl, 0.2 mM DTT, 0.2 μg/ml of each leupeptin, chymostatin, and pepstatin, 10% glycerol). Fractions containing p97 were dialyzed against buffer B (20 mM MES–NaOH (pH 6.5), 1 mM $MgCl_2$, 0.15 M NaCl, 0.2 mM DTT, 0.2 μg/ml of each leupeptin, chymostatin, and pepstatin, 10% glycerol), and applied to an SP-Sepharose column equilibrated with buffer B. The flow-through fractions were pooled, dialyzed against buffer C (20 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 0.2 mM dithiothreitol (DTT), 0.2 μg/ml of each leupeptin, chymostatin, and pepstatin, 20% glycerol) containing 50 mM NaCl, and applied to a DEAE-5PW column equilibrated with buffer C. Proteins were eluted with a linear gradient of 50 mM–2 M NaCl in buffer C. Fractions containing p97 were dialyzed against buffer G (50 mM potassium phosphate (pH 7.5), 1 mM $MgCl_2$, 0.2 mM DTT, 0.2 μg/ml of each leupeptin, chymostatin, and pepstatin, 20% glycerol), applied to an HA-1000 (hydroxylapatite) column equilibrated with buffer G, and eluted with a linear gradient of 50–800 mM potassium phosphate buffer. Glycerol was added to p97-containing fractions to a final concentration of 40% and the fraction was stored at −20°C.

2.5. Preparation of Xenopus egg extract and sperm chromatin

Xenopus egg extract and sperm chromatin was prepared as described [1].

2.6. Immunofluorescence microscopy

Samples for immunofluorescence microscopy were prepared as follows.

Sperm chromatin was incubated with egg extracts (containing 25 μM phosphocreatine, 75 μg/ml creatine kinase, 1 mM ATP, 13 μM biotin-14-dCTP) for the indicated times at 23°C to reconstitute the nuclei. Ten microliter samples were fixed with 50 μl NWB (200 mM sucrose, 15 mM HEPES–NaOH (pH 7.4), 50 mM NaCl, 2.5 mM $MgCl_2$, and 1 mM DTT) containing 3% formaldehyde. The fixed samples were overlayered onto 1 ml cushions of NWB containing 25% glycerol with coverslips under the cushion, and centrifuged at 1200×*g* in a swing rotor. The nuclei attached to the coverslips were incubated first with the anti-p97 antibody and then with FITC-conjugated goat anti-rabbit IgG and Texas red-conjugated avidin, to visualize p97 and synthesized DNA, respectively.

2.7. Chromatin-binding assay

Sperm chromatin was incubated with egg extracts containing phosphocreatine, creatine kinase, and ATP for 60 min at 23°C, and 10 μl samples were diluted with 50 μl NWB containing 0.25% Triton X-100. The samples were overlayered onto cushions of NWB containing glycerol, and centrifuged. The precipitates were recovered as chromatin fractions, and analyzed by Western blotting using anti-p97 antibody. As a control experiment, samples were incubated without sperm chromatin and processed in the same way.

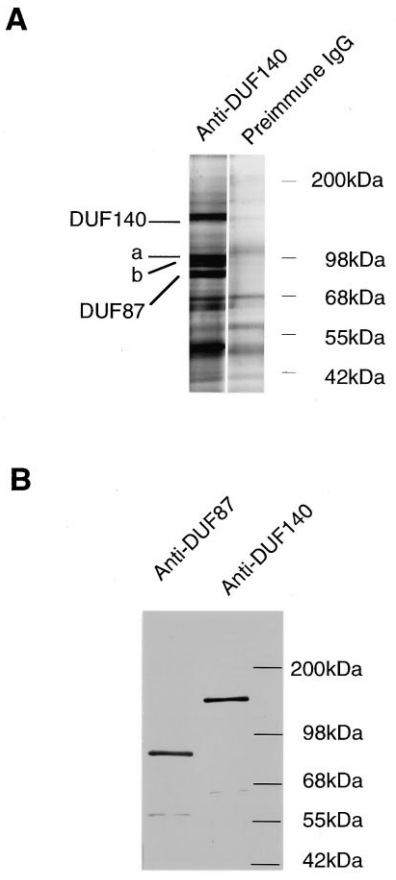

Fig. 1. Screening of DUF-interacting proteins by immunoprecipitation. A: Immunoprecipitates of *Xenopus* egg extracts with anti-DUF140 antibody (lane Anti-DUF140) and IgG purified from preimmune serum (lane Preimmune IgG) were analyzed by SDS-PAGE and silver-staining. The polypeptide corresponding to band 'a' was microsequenced. B: Western blot analysis of the precipitates formed by immunoprecipitation using anti-DIF140 antibody with anti-DUF87 (lane Anti-DUF87) and anti-DUF 140 (lane Anti-DUF140) antibodies.

3. Results and discussion

3.1. Analysis of proteins that co-immunoprecipitate with DUF

To identify proteins bound to DUF, we immunoprecipitated DUF from *Xenopus* egg extracts with anti-DUF140 antibody. Four proteins with apparent molecular masses of about 140 kDa, 100 kDa (a), 100 kDa (b), and 90 kDa were specifically recovered in the precipitate with anti-DUF140 antibody (Fig. 1A). Western blotting analysis using anti-DUF140 and anti-DUF87 antibodies indicated that the proteins with molecular masses of 140 kDa and 90 kDa were DUF140, and DUF87, respectively (Fig. 1B). Of the two proteins of about 100 kDa, we further studied the major protein (band 'a' in Fig. 1A). To identify the protein, it was subjected to microsequencing. Amino acid sequences of the peptides derived from the 100 kDa protein 'a' are shown in Fig. 2A. These were identical to partial amino acid sequences of p97 ATPase (Fig. 2B). The identity was confirmed by Western blotting with anti-p97 polyclonal antibody (Fig. 2C) and a monoclonal antibody against p97 generously provided by Dr. Jan-Michael Peters of the Research Institute of Molecular Pathology, Vienna, Austria (data not shown).

A

Peptide 1: EMDELQLFRGDTV
Peptide 2: KLSDDVDLEQVANETHG

B

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MASGSDTKSD DLSTAILKQK SRPNRLIVDE SINEDNSVVS LSQAKMDELQ      50
LFRGDTVLLK GKKRREAVCI VLSDDTCSDE KIRMNRVVRN NLRVRLGDVI     100
SIQPCPDVKY GKRVHVLPID DTVEGITGNL FEVYLKPYFL EAYRPIRKGD     150
IFLVRGGMRA VEFKVVETDP SPYCIVAPDT VIHCEGEPIK REDEEESLNE     200
VGYDDIGGCR KQLAQIKEMV ELPLRHPALF KAIGVKPPRG ILLYGPPGTG     250
KTLIARAVAN ETGAFFFLIN GPEIMSKLAG ESESNLRKAF EEAEKNAPAI     300
IFIDELDAIA PKREKTHGEV ERRIVSQLLT LMDGLKQRAH VIVMAATNRP     350
NSIDPALRRF GRFDREVDIG IPDSTGRLEI LQIHTKNMKL SDDVDLEQVA     400
NETHGHVGAD LAALCSEAAL QAIRKKMDLI DLEDETIDAE VMNSLAVTMD     450
DFRWGLSQSN PSALRETVVE VPQVTWEDIG GLEDVKRELQ ELVQYPVEHP     500
DKFLKFGMTP SKGVLFYGPP GCGKTLLAKA IANECQANFI SIKGPELLTM     550
WFGESEANVR EIFDKARQAA PCVLFFDELD SIAKARGGNI GDGGGAADRV     600
INQILTEMDG MSIKKNVFII GATNRPDIID PAILRPGRLD QLIYIPLPDE     650
KSRMAILKAN LRKSPVAKDV DVDFLAKMTN GFSGADLTEI CQRACKLAIR     700
ESIENEIRRE RDRQTNPSAM EVEEDDPVPE IRRDHFEEAM RLARRSVSDN     750
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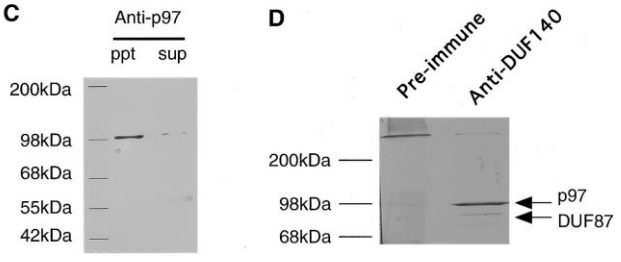

Fig. 2. Identification of the 100 kDa DUF-binding protein as p97. A: Amino acid sequences of peptides derived from the 100 kDa protein. The 100 kDa DUF-binding protein was digested with lysyl endopeptidase and the derived peptides were separated by HPLC and processed for microsequencing. The amino acid sequences of two peptides are presented. B: Complete amino acid sequence of p97 [10]. The sequences corresponding to those in A are double-underlined. C: Western blot analysis of immunoprecipitates formed by anti-DUF140 antibody with antibody against p97. Immunoprecipitates of *Xenopus* egg extract with anti-DUF140 antibody were analyzed by Western blotting using the anti-p97 antibody. The immunoprecipitate (ppt) and supernatant (sup) fractions are shown. D: Immunoprecipitation of *Xenopus* A6 cell extracts with anti-DUF140 antibody. Cell extracts prepared from *Xenopus* A6 cells were analyzed by immunoprecipitation with anti-DUF140 antiserum (lane Anti-DUF140) and pre-immune serum (lane Pre-immune). The immunoprecipitates were examined by Western blotting using anti-p97 and anti-DUF87.

As many proteins are stockpiled in eggs, non-specific protein–protein interactions might take place. To exclude this possibility, we performed immunoprecipitation with an A6 cell extract. A6 cells are an established *Xenopus* kidney cell line. Immunoblotting with anti-p97 antibody showed that p97 in the A6 cell extract was specifically immunoprecipitated by the anti-DUF140 antibody (Fig. 2D). This result indicates that DUF and p97 formed a complex in somatic cells as well as in eggs.

3.2. Purified DUF and p97 form a complex in vitro

To confirm the interaction between DUF and p97, we tested whether DUF and p97 form a complex in vitro. We purified DUF and p97 from *Xenopus* egg extract. The purification procedures for both proteins and the SDS-PAGE patterns of the purified DUF and p97 are shown in Fig. 3A,B, respectively. Purified DUF and p97 were mixed, dialyzed against NP-40 lysis buffer, and examined by immunoprecipitation with anti-DUF140 antibody. The precipitates and supernatants were analyzed by Western blotting using anti-DUF87 and anti-p97 antibodies. Fig. 3C shows that p97 and DUF87 were specifically immunoprecipitated by anti-DUF140. This result indicates that purified DUF and p97 form complexes.

3.3. p97 is incorporated into nuclei formed in Xenopus egg extracts

The subcellular localization of p97 in A6 cells has been studied by Peters et al. [12]. We confirmed their results with anti-p97 antibody prepared in this study, and found that p97 exists in both the cytoplasm and the nucleus (data not shown). We further examined the incorporation of p97 into the nucleus using a cell-free nuclear reconstitution system of *Xenopus* egg extracts. Sperm chromatin exogenously added to *Xe-*

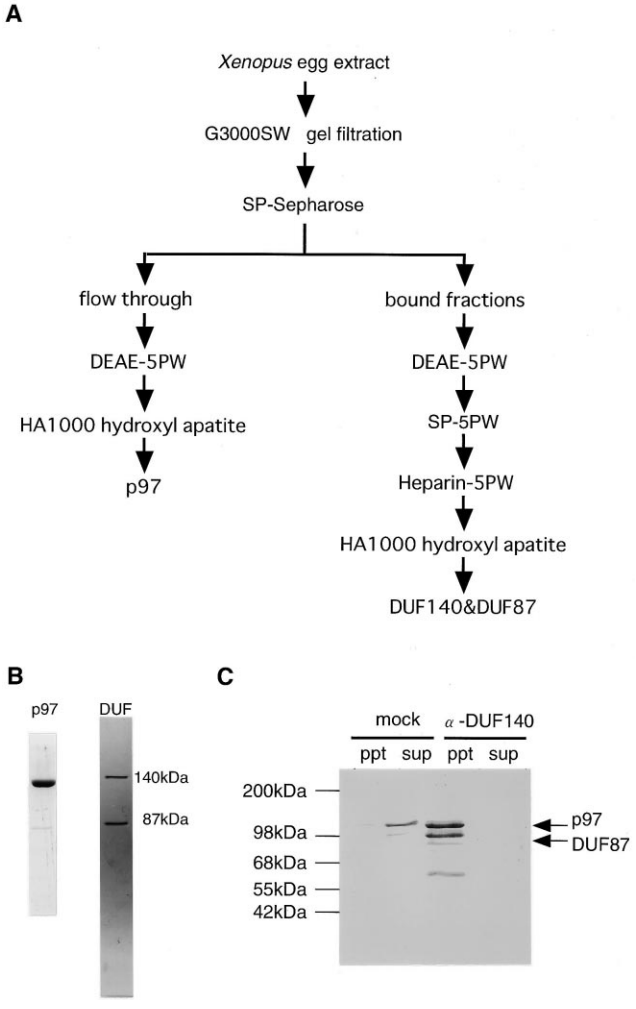

Fig. 3. Binding of DUF and p97 in vitro. A: Procedures for the purification of p97 and DUF from *Xenopus* egg extracts. B: SDS-PAGE pattern of purified DUF and p97. C: Binding of purified DUF and p97 in vitro. Purified DUF and p97 were mixed, dialyzed against NP-40 lysis buffer, and examined by immunoprecipitation using anti-DUF140 antibody (lanes α-DUF140) and pre-immune IgG (lanes mock). The immunoprecipitates (lanes ppt) and immunoprecipitation supernatant (lanes sup) were analyzed by Western blotting using a mixture of anti-DUF87 and anti-p97 antibodies.

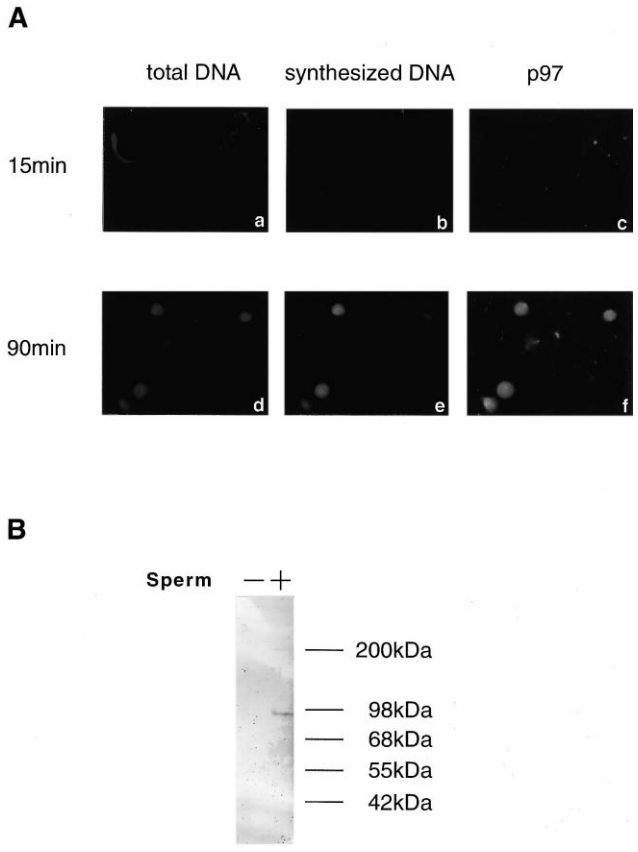

Fig. 4. Incorporation of p97 into nuclei formed in egg extracts. A: Staining of sperm nuclei assembled in *Xenopus* egg extracts after 15 min incubation (a,b,c) and 90 min incubation (d,e,f). Total DNA, newly synthesized DNA, and p97 were visualized with DAPI (a,d), biotinylated cCTP and Texas red-conjugated avidin (b,e), and anti-p97 antibody and FITC-conjugated secondary antibody (c,f), respectively. B: Chromatin binding of p97. Chromatin fractions prepared from nuclei reconstituted from sperm chromatin in *Xenopus* egg extracts (lane sperm +) and similar fractions prepared in the same experiment without sperm chromatin (lane sperm −) were analyzed by Western blotting with anti-p97 antibody.

nopus egg extracts undergoes chromatin decondensation, formation of nuclear envelope, and DNA replication [20]. As these processes proceed, various factors required for each step are recruited to the nucleus. For instance, DNA polymerase α-primase is recruited to the nucleus prior to DNA replication [21]. The same is true of DUF [1], and we examined the incorporation of p97 into the nucleus using immunofluorescence microscopy. Fig. 4A shows the sperm chromatin incubated with *Xenopus* egg extracts for 15 min and 90 min. After 15 min incubation, when nuclear formation and DNA replication had not yet started, no p97 was observed in the nuclei. When incubation was continued for 90 min, at which time nuclear formation was complete and DNA synthesis had started, it was obvious that p97 had been taken into the reconstituted nuclei undergoing DNA replication.

To examine further the status of p97 in the nucleus, we analyzed the chromatin fraction prepared by treatment of the reconstituted nuclei with Triton X-100. The chromatin-bound proteins were not solubilized with Triton X-100 and could be precipitated by centrifugation through a glycerol cushion [22]. We compared the Triton X-100-insoluble materials obtained from sperm chromatin incubated in egg extract with those obtained from mock samples without sperm chromatin. The results shown in Fig. 4B indicate that at least a part of p97 bound to chromatin.

3.4. What is the significance of the DUF–p97 interaction?

Considering the data presented, we would like to discuss the possible significance of the DUF–p97 interaction. For instance, two possibilities concerning the function of p97 can be considered. The idea that AAA ATPase may govern the formation and dissociation of the protein complex by ATP binding and hydrolysis was advocated by Confalonieri and Duguet [9]. Since DNA replication (and/or other DNA-related events) is supposed to be executed by large protein complexes and their interactions, DUF would function as part of a complex. One possibility concerning the function of p97 is that the energy from ATP hydrolysis by p97 may be required for the construction and/or destruction of a postulated DUF-containing complex. A second possibility is that p97 might function in an ATP-requiring step in the modification of chromatin structure by DUF. It has been reported that a yeast DUF140 homologue, Cdc68p, would affect chromatin structure [2,3] and our preliminary experiments showed that DUF interacts with chromatin. Indeed, chromatin-specific transcription elongation factor, FACT, which facilitates transcription elongation from the chromatin template, is a human counterpart of *Xenopus* DUF [7,8]. Therefore, it is probable that ATP hydrolysis by p97 in complex with DUF may produce a chromatin structure favorable for DNA replication. Further study is needed to elucidate the biological significance of the interaction between DUF and p97.

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References

[1] Okuhara, K., Ohta, K., Seo, H., Shioda, M., Yamada, T., Tanaka, Y., Dohmae, N., Seyama, Y., Shibata, T. and Murofushi, H. (1999) Curr. Biol. 9, 341–350.
[2] Malone, E.A., Clark, C.D., Chiang, A. and Winston, F. (1991) Mol. Cell. Biol. 11, 5710–5717.
[3] Rowley, A., Singer, R.A. and Johnston, G.C. (1991) Mol. Cell. Biol. 11, 5718–5726.
[4] Shirakata, M., Huppi, K., Usuda, S., Okazaki, K., Yoshida, K. and Sakano, H. (1991) Mol. Cell. Biol. 11, 4528–4536.
[5] Bruhn, S.L., Pil, P.M., Essigmann, J.M., Housman, D.E. and Lippard, S.J. (1992) Proc. Natl. Acad. Sci. USA 89, 2307–2311.
[6] Wittmeyer, J. and Formosa, T. (1997) Mol. Cell. Biol. 17, 4178–4190.
[7] Orphanides, G., LeRoy, G., Chang, C.-H., Luse, D.S. and Reinberg, D. (1998) Cell 92, 105–116.
[8] Orphanides, G., Wu, W.-H., Lane, W.S., Hampsey, M. and Reinberg, D. (1999) Nature 400, 284–288.
[9] Confalonieri, F. and Duguet, M. (1995) BioEssays 17, 639–650.
[10] Frohlich, K.-U., Fries, H.-W., Rudiger, R., Erdmann, D., Botstein, D.L. and Henson, P.M. (1991) J. Cell Biol. 114, 443–453.
[11] Egerton, M., Ashe, O., Chen, D., Druker, B., Burgess, W. and Samelson, L. (1992) EMBO J. 11, 3533–3540.
[12] Peters, J.-M., Walsh, M.J. and Franke, W.W. (1990) EMBO J. 9, 1757–1767.
[13] Latterich, M., Frohlich, K.-U. and Schekman, R. (1995) Cell 82, 885–893.
[14] Acharya, U., Jacobs, R., Peters, J.-M., Watson, N., Farquhar, M.G. and Malhotra, V. (1995) Cell 82, 895–904.
[15] Rabouille, C., Levine, T.P., Peters, J.-M. and Warren, G. (1995) Cell 82, 905–914.

[16] Madeo, F., Schlauer, J., Zischka, H., Mecke, D. and Frohlich, K.-U. (1998) Mol. Biol. Cell 9, 131–141.
[17] Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Iwamatsu, A. (1992) Electrophoresis 13, 142–147.
[19] Talian, J.C., Olmsted, J.B. and Goldman, R.D. (1983) J. Cell Biol. 97, 1277–1282.
[20] Lohka, M.J. and Masui, Y. (1983) Science 220, 719–721.
[21] Mimura, S. and Takisawa, H. (1998) EMBO J. 17, 5699–5707.
[22] Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H. and Nojima, H. (1995) Cell 81, 601–609.