Identification of phosphorylation sites of TOPORS and a role for phosphorylated residues in the regulation of ubiquitin and SUMO E3 ligase activity

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ABSTRACT OF THE DISSERTATION

Identification of phosphorylation sites of TOPORS and a role for phosphorylated residues in the regulation of ubiquitin and SUMO E3 ligase activity

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Dissertation Director:

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TOPORS is the first example of a protein that possesses both ubiquitin and SUMO E3 ligase activity. The ubiquitination activity maps to a conserved RING domain in the N-terminal region of the protein, which is not required for sumoylation activity. Similar to other E3 ligases, it is likely that the ubiquitin and sumoylation activities of TOPORS are regulated by post-translational modifications. Therefore, we employed mass spectrometry to identify post-translational modifications of TOPORS. Several putative phosphorylated regions were identified in conserved regions of the protein. We investigated the role of phosphorylation of serine 98, which is adjacent to the RING domain, in both cells and in vitro. Mutation of serine 98 to aspartic acid resulted in an increase in the ubiquitin ligase activity of TOPORS both in cells and in vitro. In addition, this mutation increased the binding of TOPORS to the E2 enzyme UbcH5a. Conversely, a phospho-deficient mutant (S98A) exhibited little change in ubiquitin ligase activity compared to wild-type TOPORS, both in cells and in vitro. Neither of the mutants affected the localization of TOPORS to punctate nuclear regions. In addition, neither mutant affected the SUMO ligase activity of TOPORS in cells or in vitro. Our findings indicate that phosphorylation of serine 98 regulates the ubiquitin but not the SUMO ligase activity of TOPORS, consistent with a potential binary switch function for TOPORS in protein ubiquitination versus sumoylation.

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INTRODUCTION OF THE DISSERTATION

History of TOPORS

DNA topoisomerases are a class of enzymes that alter the topology of DNA and involve in cellular processes such as DNA replication, transcription, and repair. All organisms have both type I and type II topoisomerases. Among them, mammalian cells contain one type IB topoisomerase, topoisomerase I (Top1) and two type IA topoisomerases, topoisomerase IIIa (Top3a) and topoisomerase IIIb (Top3b), as well as two type II topoisomerases, topoisomerase IIa (Top2a) and topoisomerase IIB (Top2b). Topoisomerases were found to be targets for naturally occurring antimicrobial and anticancer drugs. Camptothecins (CPTs) target mammalian type I topoisomerases, Top1. CPTs target the covalent complex between Top1 and DNA and prevent re-ligation of DNA. CPTs also induce cell cycle arrest at G2 and cell death. Although CPTs have been approved as anti-cancer agents, there are several cases in which cancer cells develop resistance to CPTs. Overexpression of efflux pumps, Top1 mutations, and post-translational modifications of Top1 by ubiquitin and SUMO-1 are some of the examples of the CPT resistance.

Several studies suggest that interactions between Top1 and other proteins also may affect cellular resistance to CPTs. In order to study the mechanisms of resistance to CPTs, our laboratory has previously identified several proteins interacting with N-terminus of Top1 by the yeast two hybrid/*in vitro* binding assays (Haluska et al., 1999). This screening identified a novel nuclear protein with an arginine-serine (RS)-rich

domain. The protein was named TOPORS (Topoisomerase 1 binding arginine and serine-rich protein.

Protein domains of TOPORS

The human *TOPORS* gene is positioned at chromosome 9p21 and consists of 3 exons and 3 introns. TOPORS is a 1045 residue protein with a predicted molecular weight of 119 kDa. Analysis of TOPORS amino acid sequence revealed several conserved domains found in other proteins. TOPORS contains a RING (Really Interesting New Gene) domain at the N-terminus between amino acids 103-144, two bipartite nuclear localization sequences (residues 616-644), 33 RS/SR dipeptide repeats, and a C-terminal lysine-rich region (residues 880-895). The RING domain of TOPORS is highly conserved among species, including mice, *C. elegans, H. sapiens* and *D. melanogaster*. RING domains are generally involved in protein-protein interactions and catalyze ubiquitin transfer during ubiquitination process. Other interesting domains in TOPORS include 5 PEST domains, commonly found in proteins that are rapidly degraded, as well as 6 consensus SUMO conjugation sites (ψKXE), where ψ is a hydrophobic amino acid, K is a lysine residue targeted for SUMO-modification, E is glutamic acid, and X is any amino acid.

TOPORS may act as a tumor suppressor in cancers.

TOPORS is identified as a topoisomerase I binding protein and is also known as LUN since the alveolar epithelium of the lung expresses high levels (Chu et al., 2001).

TOPORS mRNA and protein are widely expressed in normal adult human tissues, but not in colon adenocarcinomas or colon cancer cell lines (Saleem et al., 2004). Studies of TOPORS expression in lung and brain malignancies by other groups showed similar results, implicating TOPORS as a tumor suppressor in various malignancies (Bredel et al., 2005). The human *TOPORS* gene is located at chromosome 9p21. Furthermore, the loss-of-heterozygosity (LOH) in this region is observed in several malignant cases. Overexpression of TOPORS by transfection inhibits cellular proliferation and is associated with cell cycle arrest at the G_0/G_1 phase. Preliminary studies from our laboratory indicate that complete loss of TOPORS results in perinatal mortality and the rate of tumorigenesis in mice heterozygous for a mutant allele is increased compared to wild-type mice. This finding indicates that haploinsufficiency of *Topors* is associated with a increased rate of maligmancy in *Topors* -/- mice. In addition, murine embryonic fibroblasts lacking TOPORS showed genomic instability and increased in the rate of cellular transformation. Metaphases from *Topors* -/- cells exhibited aneuploidy, while wild-type exhibited a normal karyotype. These results suggest that *Topors* might function as a tumor suppressor by maintaining genomic stability (i.e. by functioning as a "caretaker").

Cellular localization of TOPORS

The TOPORS RING domain is similar to that of the herpesvirus protein ICP0/Vmw110, which localizes in PML nuclear bodies. Previously our group published that TOPORS localizes in punctate nuclear regions associated with PML nuclear bodies

(Rasheed et al., 2002). Fluorescent immunocytochemistry studies revealed the subcellular localization of TOPORS. Both endogenous TOPOR and exogenous TOPORS expressed as a GFP-fusion protein are localized in a punctate pattern in the nuclei of HeLa and H1299 cells, with the TOPORS foci co-localizing with promeylocytic leukemia nuclear bodies (PML NBs). PML NBs, also named ND10 or PML oncogenenic domains (PODs), are nuclear matrix-associated spherical structures that are present in varying numbers and sizes depending on the cell type and treatment of cells with various stimuli. In acute promyelocytic leukemia (PML) cells harboring a reciprocal t(15;17) translocation, the *PML* gene is fused with the retinoic acid receptor-α (*RAR*α) gene, which results in PML-RARα fusion protein and disruption of NBs. Studies using isogenic *PML* +/+ and *PML* -/- murine embryonic fibroblasts established that localization of TOPORS to PML NBs was dependent on the intact PML protein because TOPORS localized diffusely throughout the nucleus in PML -/- cells. Deletion mapping using various GFP-fusion fragments of TOPORS illustrated that the conserved N-terminal RING domain of TOPORS was not required for the punctate nuclear localization. Furthermore, TOPORS residues 1-539 or 705-1045 localized diffusely throughout the nucleoplasm, indicating that TOPORS residues 540-704 are necessary for punctate nuclear localization. Similar results were found by another group; TOPORS residues 437-555 are sufficient for punctate nuclear localization of TOPORS in HeLa cells (Weger et al., 2003).

Ubiquitination and ubiquitin-like protein modification

Post-translational modifications of proteins occur in cells in response to alterations in their external or internal environments. Post-translational modifications include phosphorylation, acetylation, and modifications by ubiquitin and ubiquitin like modifiers (UBLs). Ubiquitin exists as a precursor that is processed by de-ubiquitinating enzymes (DUBs) to generate a Gly-Gly sequence at the C-terminus that involves an isopeptide bond between the amino groups of a Lys residue on the target protein. The ubiquitination process involves three enzymatic steps. The first step is E1 (the Ub-activating enzyme) dependent adenylation of the C-terminus of ubiquitin, an ATP-dependent process, and is followed by transfer of ubiqutin to a Cys residue on the E1 via thiolester linkage. Next, ubiquitin is transferred to a Cys residue on an E2 (Ub-conjugating enzyme). The E2 transfers the activated ubiquitin to the substrate either directly or via an E3 (Ub-ligase). Ubiquitin E3 ligases function as the substrate recognition modules of the system and are capable of interaction with both E2 and substrate (Figure 18).

Ubiquitin E3 ligases possess one of two domains: HECT (Homologous to the E6-AP Carboxyl Terminus) domain or RING (or the closely related U-box domain). Ubiquitin transfer can occur in two ways: directly from E2, catalyzed by RING domain of E3 ligases or via an E3 ligase, catalyzed by HECT domain of E3. HECT E3s transfer ubiquitin from the E2 to a conserved Cys residue in the HECT domain forming an intermediate thiolester, which is attacked by substrate Lys residue (Pickart, 2004). While RING E3s do not form a thiolester intermediate with ubiquitin, they help transferring ubiquitin from the E2 to the substrate (Hershko and Ciechanover, 1998). The ‘cross-

brace' structure of RING finger creates a globular domain that can directly bind the E2s (Hershko and Ciechanover, 1998). One function of ubiquitination is to direct target proteins to 26S proteosome for degradation. Generally, 26S proteasome targeting degradation requires a formation of a polymeric ubiquitin chain on the targeted protein. The polymeric ubiquitin chain is formed by a sequential formation of ubiquitin isopeptide bond between Lys48 of one ubiquitin and the C-terminal carboxyl group of the next ubiquitin. Chains can also involve other internal ubiquitin lysines, with these chains results in distinct cellular consequences. Lys63- linked ubiquitin chains have roles in post-replication DNA repair, translation and endocytosis. Monoubiquitination of proteins also plays a role in endocytosis/lysosomal degradation, meiosis, and chromatin remodeling. In addition, Nedd4 or Itch HECT E3 ligase binds to Cbl proteins and targets them for proteasomal degradation (Magnifico et al., 2003).

There are other various ubiquitin like proteins, including small ubiqutin like modifier (SUMO), NEDD8 (RUB1) ISG15 (UCRP), AUT7 (APG8), URN1, HUB1, FAT10, MNSF and APG12. Most of them shares sequential and structural similarity with ubiquitin. SUMO shares only 18% sequence similarity but NMR studies reveal an ubiquitin-like protein fold. Vertebrates have 3 isoforms of SUMO: SUMO-1, SUMO-2 and SUMO-3. SUMO-2 is ~98% identical to SUMO-3 and is ~50% identical to SUMO-1. Although structurally similar to ubiquitin and other ubiquitin like modifiers (UBLs), the charge distribution of SUMO is very different.

Like ubiquitination, SUMOylation comprises three enzymatic steps: SUMO E1 (activating), E2 (conjugating), and E3 (ligase) enzymes. SUMO E1 exists as a

heterodimer in yeast (Aos/Aba2) and humans (SAE1/SAE2). However, both components of SUMOE1 enzyme are related to the ubiquitin E1 enzyme such that Aos1/SAE1 resembles the N-terminus of ubiquitin E1, while Uba2/SAE2 corresponds to the C-terminus and contains the active site Cys. In addition, Ubc9 is the only SUMO conjugating enzyme (E2) in yeast as well as other vertebrates, whereas there are multiple ubquitin conjugating enzymes. There are three distinct classes of SUMO E3 ligases: the RING type E3 ligases such as PIAS (Protein Inhibitor of Activated STAT) family members (Hochstrasser, 2001), RING-independent E3 ligases such as RanBP2, and Pc2 (Kirsh et al., 2002) (Kagey et al., 2003). PIAS family members sumoylate various cellular proteins including p53, LEF1 and septins (Kahyo et al., 2001; Sachdev et al., 2001; Schmidt and Muller, 2002; Takahashi et al., 2001), while RanBP2 sumoylates HDAC4 and Sp100 (Kagey et al., 2003; Kirsh et al., 2002), and Pc2 sumoylates CtBP and HIPK2 (Kagey et al., 2003; Roscic et al., 2006).

SUMO has three isoforms as mentioned before: SUMO-1,-2, and -3. The biological roles of SUMO-1 are antagonizing ubiquitination such as Iκbα and regulating protein-protein interactions, nucleocytoplasmic transport, as well as transcriptional activity of proteins, PML body formation, transcriptional repression, chromosome organization and function, and maintenance of genomic integrity (Haracska et al., 2004). SUMO-2/-3 are strongly induced by various stresses and are more abundant than SUMO-1. Recently it is reported that SUMO-2/-3 conjugation is involved in p53/Rb-induced senescence (Bischof et al., 2006) (Anckar et al., 2006).

TOPORS is the first protein with dual ubiquitin and SUMO E3 ligase activities

TOPORS contains an N-terminal C3HC4 type RING domain that is conserved in TOPORS orthologs and has similarities with the RING domains of known E3 ubiquitin ligases such as the herpesvirus protein ICP0 and CBL (Joazeiro and Weissman, 2000) (Boutell et al., 2002). RING-type E3 ligases work as a single unit or as part of a multisubunit complex and can also catalyze formation of free substrate-independent poly-ubiquitin chains (Boutell et al., 2002). Our group reported that polyubiquitin chain formation was stimulated by TOPORS in conjunction with various ubiquitin conjugating enzymes (E2s) using in vitro ubiquitination assays. A known target for ubiquitination, p53, is also a TOPORS-interacting protein, In the presence of TOPORS, p53 is ubiquitinated, demonstrated by both *in vivo* and *in vitro* assays (Rajendra et al., 2004) (Zhou et al., 1999). The *Drosophila* TOPORS (dTOPORS) ortholog ubiqutinates Hairy, a transcriptional repressor (Secombe and Parkhurst, 2004). TOPORS also ubiquitinates NKX3.1 which functions as a haploinsufficient tumor suppressor in prostate cancer (Guan et al., 2008).

In addition, it is also reported that TOPORS can act as a SUMO-1 E3 ligase for p53 and topoisomerase I (Hammer et al., 2007; Shinbo et al., 2005; Weger et al., 2005). In contrast to ubiquitination, the RING domain of TOPORS is dispensable for the SUMOylation activity (Weger et al., 2005).

Phosphorylation and other posttranslational modification regulate ubiquitin or SUMO E3 ligase activity.

Posttranslational modifications are alteration to a protein structure and are made enzymatically after proteins are translated. Many E3 ligase activities are modulated by post translational modifications such as phosphorylation, nitrosylation, SUMOylation, acetylation, and neddylation. Among them, phosphorylation prominently regulates the E3 ligase activities by creating the binding sites for E3s on substrates or ubiquitin conjugating enzymes (E2s). The ubiquitin E3 ligase activity of Siah2, COP1, and Cbl are known to be enhanced by phosphorylation (Dornan et al., 2006; Kassenbrock and Anderson, 2004; Khurana et al., 2006). A structural study of the Cbl-UbcH7 complex suggests that phosphorylation of residue Tyr371 on c-Cbl increases its ubiquitination activity by affecting E2 binding (Levkowitz et al., 1999; Yokouchi et al., 2001; Zheng et al., 2000). Phosphorylation of the c-Cbl ubiquitin ligase near the RING domain results in enhanced ubiquitin E3 ligase activity toward EGFR (Levkowitz et al., 1999). Phosphorylation has also been implicated in regulation of SUMO E3 ligase activity. Phosphorylated Ser within the SUMO-1-interacting motif (SIM) of PIAS helps to maintain specificity and orientation of binding of SUMO1 by providing additional electrostatic interactions (Hecker et al., 2006). p300/CBP acetylates the same C-terminal Lys of p53 targeted for ubiquitination by Mdm2. Thus, acetylation of the C-terminal Lys stabilizes and activates p53 (Brooks and Gu, 2003). RING E3 ligases contain Cys residues in the RING domains that may react with nitric oxide (NO) to form an S-nitrosylated derivative (Stamler et al., 1997; Wang et al., 2006). S-nitrosylation of Parkin (ubiquitin E3 ligase) by nitrosative stress initially increases autoubiquitination and then impairs its ubiquitination activity on its substrates (Yao et al., 2004).

Neddylation of Cul-1l a subunit of ubiquitin ligase complex called ROC1-SCF complex, enhances the ubiquitination activity of the SCF complex by recruiting activated E2 enzyme to SCF complex (Kawakami et al., 2001). SUMOylation on MDM2 regulates its nuclear localization (Miyauchi et al., 2002). ISG18 modification on Nedd4 interferes its ubiquitin E3 ligase activity.

Previous studies from our lab and others indicated that TOPORS may be the first protein to possess dual ubiquitin/SUMO E3 ligase activity. Many known dual enzyme activities are regulated by posttranslational modifications. My work in the laboratory has focused on the identification of posttranslational modifications and the investigation of their role in the regulation of E3 ligase activity of TOPORS.

EXPERIMENTAL PROCEDURES

Materials

Adenosine 5'-triphosphate (ATP) (Sigma (St. Louis, MO)

Agar (Difco Laboratories (Detroit, MI))

Antibodies:

- Actin monoclonal antibody (Sigma (St. Louise, MO))
- GFP monoclonal antibody (Roche (Indianapolis, IN))
- GST polyclonal antibody (Amersham-Pharmacia (Piscataway, NJ))
- GST-Topors polyclonal antibody (Biosynthesis, Inc. (Lewiswille, TX))
- HRP-conjugated anti-goat IgG (Santa Cruz Biotechnology (Santa Cruz, CA)
- HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology (Santa Cruz, CA)
- HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology (Santa Cruz, CA)
- SUMO-1 monoclonal antibody (Zymed (San Francisco, CA)
- p53 monoclonal antibody (Santa Cruz Biotechnology (Santa Cruz, CA)

Blue Juice 10 X DNA loading day (Gibco (Gaithersburg, MD))

BSA (Sigma (St. Louise, MO))

Buffers:

- 1M Tris-HCl pH7.5 (USB (Cleveland, OH))
- 1M Tris-HCl pH8.0(USB (Cleveland, OH))
- 10X TAE buffer (Bio-Rad Laboratories (Hercules, CA)

10X TG buffer (Bio-Rad Laboratories (Hercules, CA)

10X TGS buffer (Bio-Rad Laboratories (Hercules, CA)

Carbenicillin (Sigma (St. Louise, MO))

Coomassie Blue (Bio-Rad Laboratories (Hercules, CA))

Cell lines:

H1299

HEK293

Centricon-10, Centricon-30, Centricon-100 (Amicon, Inc. (Beverly, MA))

Chemically competent bacterial strains:

BLR(DE3)pLysS(Novagen, Inc., (Darmstadt, Germany))

DH5a (Invitrogen (Carlsbad, CA))

DH10B (Invitrogen (Carlsbad, CA))

XL-10 Gold ultracompetent cells (Stratagene, La Jolla, CA)

Chemiluminescence detection kit (NEN Life Science Products, Inc. (Boston, MA))

Creatine phosphate (Sigma (St. Louis, MO))

Creatine phosphokinase (Sigma (St. Louis, MO))

DMEM (Gibco BRL Life Technologies (Gaithersburg, MD))

DMSO (Sigma (St. Louis, MO))

DNA 1Kb ladder (Invitrogen life Technologies (Gaithersburg, MD))

E2 (UbcH9) protein (Sigma (St. Louis, MO))

EDTA (J.T.Baker, Inc. (Phillipsburg, NJ))

Ethanol (J.T.Baker, Inc. (Phillipsburg, NJ))

FBS (Gibco BRL Life Technologies (Gaithersburg, MD))

Glacial acetic acid (J.T.Baker, Inc. (Phillipsburg, NJ))

Glycerol (J.T.Baker, Inc. (Phillipsburg, NJ))

Glutathione –sepharose beads (Amersham-Pharmacia (Piscataway, NJ)

Guanidine Hydrochloride (Sigma (St. Louis, MO))

Imidazole (Sigma (St. Louis, MO))

Isopropanol (J.T.Baker, Inc. (Phillipsburg, NJ))

Kanamycin (Sigma (St. Louis, MO))

LB medium capsules (Bio 101, Inc. (Vista, CA))

Leupeptin (Sigma (St. Louis, MO))

Lipofectamine 2000 (Gibco BRL Life Technologies (Gaithersburg, MD))

Methanol (J.T.Baker, Inc. (Phillipsburg, NJ))

Maxiprep DNA purification kit (Sigma (St. Louis, MO))

Miniprep DNA purification kit (Qiagen, Inc. (Valencia, CA))

N-ethyl maleimide (NEM) (Sigma (St. Louis, MO))

Nickel- NTA beads (Qiagen, Inc. (Valencia, CA))

NZY broth

Pepstatin (Sigma (St. Louis, MO))

Phosphate buffered saline mix (Sigma (St. Louis, MO))

Plasmids:

pcDNA4/HisMax-Topors 1-1045 (constructed by Hye-Jin Park (Pungaliya et al., 2007))

pcDNA4/HisMax-Topors 196-1045 (constructed by Hye-Jin Park)

pEGFP-C1, pEGFP-N1 (Clontech-B.D. Biosciences, San Jose, CA)

pEGFP-Topors (constructed by Paul Haluska (Haluska et al., 1999))

pEFGP-Topors (S98A) (constructed by Hye-Jin Park)

pEFGP-Topors (S98D) (constructed by Hye-Jin Park)

pEFGP-Topors (W131A) (constructed by Hye-Jin Park)

pGEX-4T-3 (Amersham-Pharmacia (Piscataway, NJ))

pGEX-Topors 1-1045 (constructed by Zeshaan Rasheed (Rasheed et al., 2002))

pGEX-Topors (S98A) (constructed by Hye-Jin Park)

pGEX-Topors (S98D) (constructed by Hye-Jin Park)

pMT.107-His6-Ubiquitin (Dirk Bohmann, University of Rochester, NY)

pcDNA3-His6-SUMO-1 (Ronald hay, University of St. Andrews, Scotland)

PMSF (Sigma (St. Louis, MO))

Potassium chloride (J.T.Baker, Inc. (Phillipsburg, NJ))

Potassium phosphate (Sigma (St. Louis, MO))

Prestained protein marker (New England Biolabs, Inc. (Beverly, MA))

Purified recombinant proteins:

E1 (rabbit)-ubiquitin activating enzyme (Sigma (St. Louis, MO))

E1 (human)-ubiquitin activating enzyme (Boston Biochem, Boston, MA)

E2 (UbcH5a) protein (Boston Biochem, Boston, MA)

E2 (UbcH9) protein (Sigma (St. Louis, MO))

SUMO-1 (Sigma (St. Louis, MO))

Ubiquitin (Boston Biochem, Boston, MA)

Pyrophosphatase (Sigma (St. Louis, MO))

Restriction endonuclease (New England Biolabs, Inc. (Beverly, MA))

SDS (Bio-Rad Labroatories (Hercules, CA)

Silver Stain Plus kit (Bio-Rad Labroatories (Hercules, CA)

Slide-A-Lyzer 3,500/10,000 MWCO mini dialysis chambers (Fisher Scientific)

SOC media (Gibco BRL Life Technologies (Gaithersburg, MD))

Sodium acetate (J.T.Baker, Inc. (Phillipsburg, NJ))

Sodium chloride (Sigma (St.Louis, MO))

Sodium deoxycholate (Sigma (St.Louis, MO))

Sodium EDTA (J.T.Baker, Inc. (Phillipsburg, NJ))

Sodium phosphate monobasic monohydrate (J.T.Baker, Inc. (Phillipsburg, NJ))

Sodium phosphate dibasic heptahydrate (J.T.Baker, Inc. (Phillipsburg, NJ))

Tissue cluture dishes (100-mm, 60-mm, and 6 well)

Tris base (J.T.Baker, Inc. (Phillipsburg, NJ))

Triton X-100, NP-40 (Sigma (St.Louis, MO))

Trypsin (Promega (Madison, WI))

Trypsin-EDTA (Gibco BRL Life Technologies (Gaithersburg, MD))

Tween 20 (J.T.Baker, Inc. (Phillipsburg, NJ))

Zinc chloride (Sigma (St.Louis, MO))

METHODS

DNA Operations:

Agarose Gel Electrophoresis:

Different concentrations of agarose gels ranging from 0.7% to 1.2% were made in 1X TAE buffer (40mM Tris-acetate, pH8.5, 40mM EDTA), with 0.5mg/ml ethidium bromide. Higher percentage gels were used to resolve smaller (<1kb) DNA fragments and lower percentage gels were used to resolve larger (>5kb) DNA fragments. Samples containing 1X Blue Juice DNA loading dye (6.5% sucrose, 1mM Tris-HCl, pH7.5,1mM EDTA, 0.03% bromophenol blue) were loaded into the agarose gel and electrophoresed in 1X TAE buffer at 100-120V. Fragments were visualized using a UV transilluminator and analyzed by the GelDoc program.

Plasmid DNA Purification from Bacteria

Small Scale Plasmid DNA Preparation (Qiagen Miniprep Protocol)

Bacterial cells, transformed with expression plasmids, were picked from glycerol stocks and streaked on LB-agar plates containing the appropriate antibiotic (50mg/ml carbenicillin or 30mg/ml kanamycin) and incubated at 37˚C overnight. A single bacterial colony was added to a culture tube containing 2mL LB media with appropriate antibiotic as describe above. The culture was incubated at 37˚C overnight with shaking at approximately 225rpm. The culture was transferred to a 1.5ml microfuge tube and spun

at 10,000rpm (benchtop centrifuge) for 3 minute to pellet the cells. The supernatant was discarded and pellet was resuspended in 250ml of P1 (resuspension) buffer (50mM Tris HCl, pH 8.0, 10mM EDTA, 100mg/ml RNAse A). Then P2 (lysis) buffer (200mM NaOH, 1% SDS) was added and the tubes were inverted 4-6 times, followed by the addition of 300ml of N3 (neutralization) buffer (3.0M potassium acetate, pH 5.5). The samples were inverted 4-6 times and centrifuged at 14,000 rpm for 10 minutes. The supernatant was transferred to a QIAprep spin column (silica gel matrix) and spun at 10,000 rpm for 30sec. After discarding the flow-through, the columns were washed with 750ml of PE buffer. Plasmid DNA was eluted from the column by adding 35µl TE buffer and collected by centrifugation at 13,000 rpm.

Large-Scale Plasmid DNA preparation:

Sigma Maxiprep Protocol:

A single bacterial colony was added to a culture tube with 2ml LB media and appropriate antibiotic and incubated at 37˚C for 8 hours. The culture was expanded in a 250ml culture overnight at 37˚C. The bacteria were transferred to Oak Ridge tubes (Nalgene) and then pelleted by centrifugation at 3200 rpm (Sorvall centrifuge) for 20 minutes at 4˚C. Cells were resuspended in Resuspension Solution with the appropriate volume of RNase A Solution. Cells were lysed by adding 12ml Lysis Solution. Samples ere mixed gently by inverting 6 to 8 times. The lysed cells were neutralized by adding 12ml of chilled Neutralization Solution P. Samples were inverted 4 to 6 times. 9ml Binding Solution G is added to lysed cells. Samples were inverted 1 to 2 times. Once a

filter syringe is prepared by removing a plunger, contents were poured immediately into the barrel of the filter syringe. Binding column is prepared by placing a binding column into a collection tube. After 12ml Column Preparation Solution was added to the Binding column, samples were spun in a swinging bucket rotor at 3600 rpm for 3 minutes. Lysates were filtered and DNA was bound to the column. After 12ml Wash Solution 1 was added to the column, samples were spun. After 12ml of Wash Solution 2, which should contain appropriate volume of ethanol, the samples were spun again. Plasmid DNA was eluted by transferring binding column to a new collection tube and adding 3ml Elution Solution. The samples were spun at 1800 rpm for 5 minutes. Finally, once 3ml of DNA was recovered, 300 µl 3M sodium acetate, pH 5.2 and 2.1ml of isopropanol were added to precipitate DNA. The samples were mixed well and centrifuged at 15,000xg at 4°C for 30 minutes. Supernatant was removed and cell pellets were rinsed by adding 1.5ml of 70% ethanol. The supernatant was removed and the tube was dried until the residual ethanol evaporated. Pelleted DNA was resuspended in the molecular biology grade water.

Plasmid Construction:

Prokaryotic expression plasmids

The plasmids, pGEX-TOPORS and pGEX-TOPORS (196-1045) were constructed by A. Rasheed and have been described previously (Rasheed et al., 2002). Briefly, pGEX-TOPORS, a plasmid expressing a GST-N terminal TOPORS fusion protein was constructed by using PCR amplification methods to amplify the entire coding sequence

of TOPORS from pEGFP-TOPORS and inserting it into between *EcoR I* and *Not I* sites of the pGEX-4T3 vector (Amersham Biosciences). A plasmid expressing an N-terminal polyhistidine tagged-TOPORS fusion protein (pET-TOPORS) was constructed by H. Marshall (Rasheed et al., 2002) by digestion of pKG-TOPOR with *Sma I* and *Hind III*, followed by ligation into the pET-28a (+) vector (Novagen). All plasmids were sequenced to confirm if the correct recombinants had been obtained.

Construction of pGEX-TOPORS (S98A) and TOPORS (S98D) mutants

Serine 98 in GST-TOPORS expression vector was mutated to Ala or Asp using site-directed mutagenesis (Strategene). PCRs were carried out in 50µl in the presence of 10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH8.8), 2mM $MgSO_4$, 0.1% Triton X-100, 0.1mg/ml nuclease-free bovine serum albumin (BSA), 10ng of dsDNA template (pGEX-TOPORS), 125ng of each primer, 1µl of dNTP mix, 3ul of QuikSolution, and 2.5units/µl. of *pfu Turbo* DNA polymerase PCR cycles were 1 cycle of 95ºC for 1 minute, followed by 18 cycles of 95ºC for 50 seconds, 60ºC for 50 seconds and 68ºC for 9 minutes, and 1 cycle of 68ºC for 7 minutes. The entire PCR product was subjected to 1-2 hour digestion with *Dpn I* endonuclease at 37ºC to digest methylated or hemi-methylated parental pGEX-TOPORS template. The newly synthesized nicked vector DNA incorporated in the mutations was transformed into XL10- Gold Ultracompetent cells, which repair the nicks to give rise to circular double stranded DNA. The entire coding region of TOPORS in the mutant vector was sequenced to rule out any other mutations that may have been acquired during the mutagenesis PCR.

The following primers for alanine (S98A) mutation were used: sense, 5'- CCG GCT GATG CAG CTC CTG ATT CTA AGT GTC C-3' and antisense, 5'-GGA CAC TTA GAA TCA GGA GCT GCA TCA GCC GG-3'. The following primers for aspartic acid (S98D) mutation were used: sense, 5'- CCA GCT GAT GCA GAT CCT GAT TCT AAG TGT CC-3' and antisense, 5'-GGA CAC TTA GAA TCA GGA TCT GCA TCA GCT GG-3'.

Mammalian expression plasmids:

A mammalian vector expressing the full-length TOPORS protein linked to the C-terminus of GFP (pEGFP-TOPORS) was created by P. Haluska in pEGFP-C1 plasmid (clontech BD Biosciences). A mammalian expression vector for polyhistidine-tagged TOPORS was created by ligating the TOPORS cDNA from pGEX-TOPORS into the *EcoR I* and *Not I* sites of pcDNA4-HisMax B (Invitrogen). Similarly, pcDNA-TOPORS (196-1045) was created by using pGEX-TOPORS (196-1045) and pcDNA4-HisMax C (Invitrogen), using *BamH I* and *Not I* sites. Expression plasmids for polyhistidine-tagged ubiquitin (pMT.107) and polyhistidine-tagged SUMO-1 (pcDNA3-His_6-SUMO-1) were obtained from Dirk Bohmann (University of Rochester school of medicine and dentistry, NY, USA) and Ronald Hay (University of St. Andrews, UK), respecttively.

Construction of pEGFP-TOPORS (S98A) and TOPORS (S98D) mutants

Serine 98 in the GFP-TOPORS expression vector was mutated to Ala and Asp using site-directed mutagenesis (strategene). The site-directed mutagenesis was carried as as mentioned previously for pGEX-TOPORS.

Bacterial Operations:

Bacterial Transformation

Plasmids bearing ampicillin/carbenicillin resistance gene were transformed in DH5α strain (Invitrogen) and plasmids carrying kanamycin resistance gene were transformed into DH10B or HB101 strains (Invitrogen). For the expression and purification of recombinant proteins from bacteria, the prokaryotic expression plasmids were transformed in a protease deficient *E. coli* strain, BLR(DE3)LysS (Novagen/ Calbiochem). Approximately 100ng of plasmid DNA was added to a pre-chilled sterile 14ml poly-propylene tube. 100µl of freshly thawed chemically competent bacteria were added to the tube and the mixture was incubated on ice for 30 minutes. The mixture was placed in a 42˚C water bath for 45 seconds, followed by incubation on ice for 2 minutes. Then, 900µl of SOC media was added to the bacterial culture and incubated at 37˚C for 1 hour. For BLR(DE3) LysS strain transformation, 25µl of competent cells were incubated with 10-50ng of DNA on ice for 10 minutes, followed by a 42˚C water bath incubation for 30 seconds and further incubation on ice for 2 minutes. 50µl of SOC medium was added and incubated at 37˚C for 1 hour. After transformation in any bacterial strain, 100µl of culture in SOC media was streaked on LB plates containing the appropriate antibiotic (50µg/ml carbenicillin or 30µg/ml kanamycin) and the plates were

incubated at 37˚C overnight. For XL10-Gold Ultracompetent strain transformation, 50µl of competent cells were incubated with 1µl of DNA on ice for 45 minutes. Before incubation, cells were swirled on ice every two minute for 10 minutes. After 30 seconds of 42˚C water bath incubation, 2 minute incubation on ice was followed. 100µl of NZY broth medium was added and incubated at 37˚C for 1 hour. A 2ml of miniculture was grown from a single colony picked from the plate and made for plasmid glycerol stocks, which were store in -80˚C.

Bacterial growth conditions

Cells from a glycerol stock were streaked on LB-agar plates with the appropriate antibiotic (50µg/ml carbenicillin or 30µg/ml kanamycin) and incubated at 37˚C overnight. Cells were grown at 37˚C while shaking at approximately 225rpm in LB media with the appropriate antibiotic mentioned above.

Lac-Inducible Expression of Recombinant Proteins

The protease deficient bacterial strain (BLR (DE3) LysS; Novagen/ Calbiochem) was used for the expression of recombinant proteins. For Lac-inducible expression of recombinant proteins, bacteria were grown in selective media overnight at 37˚C until reaching OD_{600} 0.7-0.9. Protein expression was induced by adding 0.1mM IPTG. The bacteria were grown for an additional 3 hours and then collected and lysed; recombinant proteins were affinity purified as described below. For GST-tagged TOPORS (wild type as well as mutants) and His-tagged TOPORS expression, the bacterial minicultures were

expanded overnight at 28˚C until OD_{600} 0.7-0.9, and then induced with IPTG at 28˚C for 3 hours before collecting.

Protein Preparation and Analysis:

GST-tagged TOPORS protein purification from bacteria

For purification of GST-tagged proteins from bacterial cell pellets, the pellets were lysed in 5X the bed volume of lysis buffer A(800mM NaCl, 50mM tris, pH 7.4, 0.5% NP-40, 5mM MgCl2, 1mg/ml lysozyme, 5mM DTT, and protease inhibitors such as 5μg/ml leupeptin, 2mM PMSF, 1μg/ml pepstatin) or lysis buffer B(50mM HEPES, pH8.0, 1mM EDTA, 1% NP40, 500mM NaCl, and protease inhibitors such as 5μg/ml leupeptin, 2mM PMSF, 1μg/ml pepstatin). The lysed bacterial cells were sonicated and spun at 12,000rpm for 20 minutes at 4˚C. The supernatant collected after the spin was incubated with equilibrated glutathione-sepharose (GSH) beads (Amersham-Pharmacia Biotech) for 2 hours or overnight at 4˚C using a rotary shaker. Following binding, the beads were washed three times with the lysis buffer without the protease inhibitors. Proteins bound to beads were stored at 4˚C in an equal bed volume of lysis buffer. For biochemical study, proteins used either bound to GSH beads or after elution. Bound proteins were eluted in 10X bed volume of elution buffer (75mM HEPES, pH8.0, 200mM NaCl, and 50mM glutathion) by vortexing at 4˚C for 45 minutes. To remove excess glutathione from the proteins, eluted GST-tagged proteins were placed in Microcon-10 sample reservoir in a vial (Amicon) and centrifuged at 14,000xg for 50 minutes. After centrifugation, the sample reservoir was placed upside down in a new vial and then spun

3 minutes at 1000xg at 4˚C to transfer concentrates to a vial. Aliquots of the protein were stored at -80˚C. The purity and concentration of purified proteins were analyzed by the silver stain before using for *in vitro* assays.

His-tagged TOPORS protein purification from bacteria

For purification of His-tagged TOPORS, pET-TOPORS was transformed into BLR(DE3) bacteria strain. After 24 hour transfection, cells were lysed with buffer A (6M guanidine HCL, 0.1% Nonidet P-40, 10mM β-mercaptoethanol, and 5% glycerol, in phosphate buffered saline, pH 8.0). Then cell lysates with 20mM (final concentration) imidazole were incubated with Nickel beads on the rotor for 4 hours at 4°C. Beads with His-tagged TOPORS protein were obtained by centrifugation at 14000rpm for 2 minutes and washed twice each with 500µl of buffer A (6M guanidine HCL, 0.1% Nonidet P-40, 10mM b-mercaptoethanol, and 5% glycerol, in phosphate buffered saline, pH8.0), 500µl of a 1:2 mixture of buffer A and buffer B (phosphate-buffered saline, 0.1% Nonidet P-40, 5% glycerol, and 20mM imidazole), 500 µl of a 1:3 mixture of buffer A and B, and 500 µl of buffer B. Bound proteins were eluted by boiling the beads in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, and 0.1% phenol red) followed by SDS-PAGE and Coomassie blue staining.

***In vitro* assays:**

***In vitro* ubiquitination assay**

In vitro ubiquitination reactions were carried out in a buffer containing 50mM HEPES, pH8.0, 5mM $MgCl_2$, 15uM $ZnCl_2$ in the presence of 4mM ATP and an ATP regenerating system (10mM creatine phosphate (Sigma), 3.5units/ml creatine kinase (Sigma), and 0.6 units/ml inorganic pyrophosphatase (Sigma)). The reactions included 150nM of human E1 (Boston Biochem) or rabbit E1 (Sigma), 300nM UbcH5a (Boston Biochem), 25μM of ubiquitin (Boston Biochem), and 3nM of purifier GST-TOPORS (wild-type or mutants). *In vitro* reactions were carried out at 30˚C for 15 minutes in a 30μL volume for 15 minutes and were terminated by addition of SDS sample buffer (60mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.1% phenol red) containing 0.1M DTT. Reaction products were resolved in 4-15% gradient SDS-PAGE gels, transferred to nictrocellulose membranes and analyzed by immunoblotting using monoclonal anti-ubiquitin (Santa Cruz Biotechnology) antibodies.

In Vitro dual ubiqutin/SUMOylation assay

To minimize effects of differential protein concentration on enzymatic assays, we performed ubiquitination and SUMOylation reactions in the same tube. The reactions contained 50mM HEPES, pH 8.0, 5mM $MgCl_2$, 15 μM $ZnCl_2$, 4mM ATP with an ATP-regenerating system (10mM creatine phosphate (Sigma), 3.5units/ml creatine kinase (Sigma), and 0.6 units/ml inorganic pyrophosphatase (Sigma)). The reactions contained 3nM of purified GST-TOPORS (wild-type or mutants), 150nM human UBE1 (Boston Biochem), 150nM SAE2/SAE1 (Sigma), 300nM UbcH5a (Boston Biochem), 300nM UbcH9 (Sigma), 25μM of ubiquitin (Boston Biochem), and 25 μM SUMO-1 (Boston

Biochem). Reactions were carried out at 30˚C for 30 minutes in a 30 µl volume and were terminated by addition of SDS sample buffer (60mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.1% phenol red) containing 0.1M DTT. Reaction products were resolved by SDS-PAGE and analyzed by immunoblotting using monoclonal anti-ubiquitin (Santa Cruz Biotechnology) or anti-SUMO-1 (Zymed) antibodies.

Mammalian Cell operations:

Cell culture

H1299 (human non-small cell lung carcinoma cells lacking endogenous p53) and HEK 293 (human embryonic kidney cells) were maintained in Dulbecco's modified Eagle's media (Gibco BRL) supplemented with 10% fetal bovine serum and 2% antibiotics (penicillin-streptomycin) at 37˚C in the presence of 5% CO_2.

His-tagged protein purification from eukaryotes

pcDNAHisMax-TOPORS was transfected into ten 10cm of H1299 cells.using lipofectamine 2000 (Gibco HRL) reagents according to the manufacturer's protocol. 24 hour after transfection, cells were lysed with buffer A (6M guanidine HCL, 0.1% Nonidet P-40, 10mM β-mercaptoethanol, and 5% glycerol, in phosphate buffered saline, pH8.0). Then cell lysates with 20mM (final concentration) imidazole were incubated with Nickel beads on the rotor for 4 hours at 4°C. Beads with His-tagged TOPORS protein were obtained by centrifugation at 14000rpm for 2 minutes and washed twice each with 500µl of buffer A (6M guanidine HCL, 0.1% Nonidet P-40, 10mM β-

mercaptoethanol, and 5% glycerol, in phosphate buffered saline, pH8.0), 500µl of a 1:2 mixture of buffer A and buffer B (phosphate-buffered saline, 0.1% Nonidet P-40, 5% glycerol, and 20mM imidazole), 500 µl of a 1:3 mixture of buffer A and B, and 500 µl of buffer B. Bound proteins were eluted by boiling the beads in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, and 0.1% phenol red). Lysates were analyzed by SDS-PAGE and immunoblotting with ubiquitin (P4D1; Santa Cruz Biotechnology), SUMO-1 (Zymed), TOPORS (Rasheed et al., 2002) or p53 (DO-1, Santa Cruz Biotechnology) antibodies.

Cellular ubiquitination and SUMOylation assays

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Equal numbers of growing cells were seeded into 6-well plates and transfected using Lipofectamine 2000 (Invitrogen) in the presence of serum- and antibiotic-free media. For ubiquitination assays, GFP tagged TOPORS were co-transfected with or without His tagged ubiquitin in HEK 293 cells. For examples, cells were transfected with 2.5µg of pEGFP-TOPORS, pEGFP-TOPORS (S98A) or pEGFP-TOPORS (98D) in the presence or absence of 500ng pMT.107 (expressing His-tagged ubiquitin). For SUMOylation assays, cells were transfected with 2.5µg of pEGFP-TOPORS, pEGFP-TOPORS (S98A) or pEGFP-TOPORS (98D) in the presence or absence of 500ng pcDNA3-His-SUMO-1 (expressing His-tagged SUMO-1). 24 hours after transfection, cells were incubated in presence or absence of 4 µM MG132 for additional 8 hours. Cells were either lysed in SDS sample-loading buffer (60 mM

Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, and 0.1% phenol red). or lysis buffer (20mM Tris-HCl (pH7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM NA_3VO4, 1ug/ml leupeptin). When cells were lysed by lysis buffer, insoluble material was required to be removed by centrifugation at 14000xg for 10 minutes at 4˚C. The supernatent was subjected for the BCA protein assay to determine the concentration in the purpose of loading equal amount of protein for this experiment.

Lysates were analyzed by immunoblotting with anti-ubiquitin (Santa Cruz), SUMO-1 (Zymed), TOPORS, and β-actin (cell signaling) antibodies.

Phosphatase treatment

After 24 hours transfection with pcDNAHisMAX-TOPORS, transfected cells were lysed with lysis buffer (50mM Tris-HCl (pH 8.0), 100mM NaCl, 0.4% NP40, 1mg/ml leupeptin, 1mg/ml pepstatin, and 1mg/ml PMSF) and sonicated for 30 seconds, followed by centrifugation for 1 minute at 14000xg. 60 units/ml of calf intestinal phosphatase (CIP: New England Biolabs) were added to 30μl of cell lysate with NEBuffer 3 (other 30μl of cell lysate served as a control). Then, cell lysates were incubated at 37ºC for 2 hours. The lysates were subjected to 5% SDS-PAGE and immunoblotting using TOPORS polyclonal antibodies. As a positive control, RS-rich splicing protein ASF/SF2 in untreated and phosphatase treated cell lysates were used with a monoclonal SF2 antibody (Zymed).

Flurescence microscopy

HEK 293 cells were grown in glass cover slips and transfected with GFP-Topors wild-type or Topors S98A and S98D mutants. After 24hour transfection, cells were fixed in 3.7% paraformaldehyde in PBS. An Eclipse TE2000 inverted fluorescence microscope equipped with a TE-FM Epi-fluorescence attachment (Nikon) and a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) was used to visualize GFP proteins in cells grown in culture dishes. GFP fluorescence will be imaged using a GFP filter set (Chroma Technology, Brattleboro, VT) with excitation and emission wavelengths of 450–490 and >500 nm, respectively.

Protein Preparation and Analysis:

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples in 1X SDS sample buffer (62.5mM Tris, pH6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 42mM DTT) were loaded onto a polyacrylamide resolving gel containing 7-10% polyacrylamide (in 375mM Tris, pH8.8, 0.l% SDS, 0.07% APS, and 0.07% TEMED) and a 4% stacking gel (in 125mM Tris-HCl, pH6.8, 0.1% SDS,0.05% APS and 0.05% TEMED) or a pre-made 5% or 4-15% gradient gel. The gel was run at 95-150V in running buffer (25mM Tris base, 190mM Glycine, 0.1% SDS) until the dye ran off the gel.

Western blot

After SDS-PAGE, proteins were transferred from the gel to a nictrocellulose membrane. The transfer was conducted at 4°C using a transfer apparatus (Bio-Rad) submerged in a transfer buffer (25mM Tris-base, 190mM glycine, 20% methanol) for 90 minutes at 60V or overnight at 20V. The nitrocellulose membranes were blocked with 5% nonfat dry milk in PBST (PBS with 0.1% Tween-20) for 1 hour at room temperature or overnight at 4°C. The blots were incubated with the desired primary antibody diluted in PBST or PBST containing 5% nonfat dry milk for 1 hour at room temperature or overnight at 4°C. The blots were washed twice for 10 minutes each with PBST and then incubated with diluted HRP-conjugated secondary antibody for 45 minutes. Finally the blots were washed in PBST twice for 10 minutes each and then developed by adding equal volumes of ECL reagents A (Perkin Elmer; Enhanced Luminal Reagent) and B (Perkin Elmer; oxidizing Reagent) and incubating for 1 minute. The blots were exposed to autoradiography film.

Silver Staining (Bio-Rad)

The purified protein samples were resolved by SDS-PAGE along with known concentrations of BSA. After gel electrophoresis, the gels were fixed in a solution (50% methanol, 10% acetic acid, 5% Fixative Enhancer Concentrate (Bio-Rad)) for at least 30 minutes. The gels were rinsed with water for 15 minutes twice. The gels were incubated in freshly prepared stain solution (5% silver complex solution, 5% reduction moderator solution, 5% image development reagent and 50% development accelerator solution)

until the bands were detected. The staining reactions were stopped by the addition of 5% acetic acid.

Mass spectrometric analysis

After resolving the purified His-tagged protein from pcDNAHis-Max TOPORS transfected HEK 293 cells, the gel was stained with Coomassie blue. Low mobility bands from a Coomassie-stained gel were cut and digested with 0.2 μg of modified trypsin (Promega) in 10 mM NH_4HCO_3 overnight at 37°C. In-gel trypsin digest samples were analyzed by nanoLC-MSMS. Samples were loaded onto a fritless nano-scale column (75um X 15 cm) self-packed with 3μm Aqua C18 material (Phenomenex, Ventura, CA). Peptides were eluted on a 30-minute linear gradient from 2% to 45% Acetonitrile in 0.1% formic acid with a flowrate of 250 nl/min using an Ultimate nano-LC system (Dionex/LC Packings, Amsterdam, The Netherlands). Eluted peptides were directly analyzed by ESI-MS/MS using a LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nanospray source (Proxeon Biosystems, Odense, Demark). Each MS scan was followed by subsequent zoom scans and MS/MS scans of the four most abundant ions, with a dynamic exclusion of 30-seccond and repeat count of two. DTA files for MS/MS spectra were generated by Bioworks software (ThermoFinnigan, San Jose, CA); results were searched using the GPM (www.thegpm.org) (Craig et al, 2004) and MASCOT search engine (www.matrixscience.com) against NCBI nr human database with fixed modification of carbamoylethylation on Cys and variable modification of phosphorylation (SPY) and

oxidation of Met allowing one missed cleavage. Point mutations, unanticipated cleavage, methionine oxidation, deamidation at asparagine and glutamine residues were allowed during model refinement. Fragment mass error was set at +/- 0.8Da, and parent mass tolerance was set at +/-2Da.

Section 1.

Identification of post-translational modification sites on TOPORS

INTRODUCTION

TOPORS is the first example protein with both ubiquitin and SUMO E3 ligase activities (Rajendra et al., 2004; Weger et al., 2005). However, the regulation of TOPORS as a dual E3 ligase has not been studied previously. However, the regulation of TOPORS as a dual E3 ligase has not understood yet. It was noted that many E3 ligase activities are regulated by posttranslational modifications. Types of posttranslational modifications regulating E3 ligase activities include phosphorylation, acetylation, ubiquitination and SUMOylation. In many ubiquitin E3 ligases, including Siah2, COP1, and Cbl, phosphorylation regulates their ubiquitin E3 ligase activity (Khurana et al., 2006). Phosphorylation of the c-Cbl ubiquitin ligase near the RING domain results in enhanced ubiquitin E3 ligase activity (Levkowitz et al., 1999). Sumolyation and acetylation are also implicated in the regulation of ubiquitin E3 ligases. Mdm2, a ubiquitin E3 ligase, ubiquitinates p53 and itself (Stad et al., 2001). SUMOylated MDM2 downregulates its ubiquitination activity and thus results in p53 accumulation (Buschmann et al., 2000; Fang et al., 2000). Acetylation on MDM2 inactivates MDM2 ligase activity toward p53 (Wang et al., 2004).

Phosphorylation and other modifications have also been implicated in the regulation of SUMO E3 ligase activity. Phosphorylation of serine residues within the acidic domain of the SUMO-1 interacting motif (SIM motif) of PIAS helps to maintain specificity and orientation of binding the lysine residue of SUMO-1 by providing

additional electrostatic interactions (Hecker et al., 2006). SUMOylation of PIASy at Lys35 is important for its nuclear localization and regulation of the activity of Tcf-4 (Ihara et al., 2005).

Previous work suggests the existence of posttranslational modifications of TOPORS in mammalian cells. It is noted that bacterially expressed TOPORS migrates at ~110Mr, while eukaryotic expressed TOPORS migrated at ~150Mr (Rajendra et al., 2004). Altered mobility due to Ser phosphorylation has been reported for RS-rich splicing factors (Fu, 1995) (Boucher et al., 2001), and it has been hypothesized that phosphorylation of the 33 RS or SR dipeptide repeats in TOPORS, several of which are conserved among various orthologs, is responsible for this migration shift. In addition, TOPORS contains 6 SUMO-1 consensus sites (ψKxE, where ψ is a hydrophobic amino acid and X is any amino acid), which suggests that TOPORS might be SUMOylated. Indeed, SUMOylation of TOPORS has been reported in HeLa cells (Weger et al., 2003).

In general, in order to identify post-translational modifications of proteins, several approaches can be utilized, including mass spectrometry, 2D gel analysis, sequencing by Edman degradation, and computational prediction (Hjerrild and Gammeltoft, 2006; Hjerrild et al., 2004). Among them, mass spectrometry is the most direct, broad, unbiased, and sensitive approach to determine the site of post-translational modifications (Knuesel et al., 2005). This approach is particularly useful for small sample quantities to minimize sample loss and identify posttranslational modifications in a short time by generating hundreds of mass spectra. Post translational modifications can be identified

by the enumeration and scoring of all possible modifications for each peptide from the database. (Beausoleil et al., 2006)

Among post-translational modifications such as phosphorylation, ubiquitination, SUMOylation and acetylation, phosphorylation events add 80Da to amino acid residue masses on Ser, Thr, and Tyr. Strategies for enriching phosphopeptides and proteins include affinity purification based on charge properties and antibody recognition. A method widely used for affinity-based purification is immobilized metal affinity chromatography (IMAC), which adsorbs phosphopeptides to chelated metal ions (Fe^{3+}, Ga^{3+}) through metal-phosphate ion pair interactions. Another method is immunopurification using highly specific antibodies to phosphotyrosine, which selects phosphotyrosine containing phosphopeptides as well as phosphoproteins. With regard to ubiquitination, trypsin digested ubiquitin results in a branched C-terminal Gly-Gly dipeptides on the modified Lys, creating a signature mass shift of 114.1Da. However, trypsin cleavage can be suppressed by Gly-Gly coupled lysine, producing missed cleavage peptides that are larger than the typical mass range of mass spectrometry (Mikesh et al., 2006). Trypsin digestion of SUMO-1 modification on Lys yields the signature mass shift of 484.2Da resulted from EQIGG remnants on the moditied lysine in yeast. However, it may be very difficult to be applied to SUMOylation site identification in mammalian cells due to the absence of Arg or Lys adjacent to the di-Gly motif (Pedrioli et al., 2006). Acetylation at Lys or N-terminal groups can be detected by its characteristic mass shift of 42.01 Da from unmodified forms. Tryptic

digestion at acetyl-lysine residues is usually blocked due to charge neutralization, so the acetylated peptides are detected as missed cleavage products (Garcia et al., 2007).

Since a number of studies have reported that post translational modifications on E3 ligases regulate their enzymatic activity, it will be focused in identifying the potential post-translational modifications of TOPORS using mass spectrometry analysis in this section.

RESULTS

Investigation of posttranslational modification on TOPORS by LC-MS/MS

To identify posttranslational modifications on TOPORS, a mass spectrometric approach is employed. As endogenous TOPORS in cells is hard to detect, polyhistidine tagged TOPORS were overexpressed in H1299 cells and *E. coli* and the tagged proteins were purified using nickel-based affinity chromatography. To decrease nonspecific binding, Ni beads were incubated with the His analog, imidazole. Purified proteins were subjected to SDS-PAGE and analyzed by both immunoblotting and Coomassie blue staining. From the Coomassie gels, 1.5mm gel slices representing TOPORS bands, identified by immunoblotting (Figure 1), were in-gel digested with trypsin, Glu-C, or Asp-N, and analyzed by LC-MS/MS. The LC-MS/MS data were analyzed using MASCOT search algorithm against human nr database. Spectra that were identified as with potential modifications were manually interpreted. (Table 1). Both phosphorylated and the corresponding non-phosphorylated peptides were detected in H1299 cells (upper panel), whereas in bacteria (lower panel), only non-phosphorylated peptides were identified (Table 1). Four phosphorylated residues were identified: Ser98, Ser499, Ser585, and Ser866. They are conserved in TOPORS orthologs in various species with a MASCOT cutoff score of 30. In addition, the MS/MS spectra corresponding to the four phosphorylated residues suggested by MASCOT were manually evaluated. (Table 1).

To identify posttranslational modifications of TOPORS, it is important to obtain good protein sequence coverage, which was improved by multiple enzyme digestion using trypsin, Glu-C and Asn-N proteolysis (Choudhary et al., 2003). Tryptic digestion (cut at C-terminal of Lys and Arg residues) of TOPORS yielded an overall protein sequence coverage 43%. TOPORS was further digested with the protease Asp-N, which cleaves N-terminal to Asp and Glu residues. LC-MS/MS analysis of this digestion resulted in an overall 9% coverage. After Glu-C digestion (cut at C-terminal of Asp and Glu residues), LC-MS/MS analysis yielded protein sequence coverage of 14%. Combining TOPORS protein coverages after each individual protease digestion resulted in approximately 60% protein sequence coverage (Figure 2).

To minimize the false positives, a minimum MASCOT score of 30 per peptide was applied. If a peptide is phosphorylated, an increase by 80Da is observed due to an attachment of the phosphate group. For example, the presence of y4 (m/z 446.36) and y5 ion peaks from phosphorylated (m/z 613.22) peptide ^{89}LQQTVPADASPDSK^{102} localizes a phosphorylation site on Ser98. Pairs of y5 ions from non-phosphorylated (m/z 533.31) and corresponding phosphorylated (m/z 613.22) peptide ^{89}LQQTVPADASPDSK^{102}that differ in mass by 80 Da provide phosphorylation on Ser98 (Figure 3). The presence of b8 (m/z 833.34) and b9 (m/z 1036.49) ions indicates a phosphorylation site on Ser499 in ^{491}TPELVELSSDSEDLGSYEK^{509} peptide (Figure 4). Peptides containing phosphorylated Ser and Thr often undergo loss of H_3PO_4 as a neutral species (‘neutral loss’), yielding a mass decreased by 98Da. y8 (m/z 936.47) and y9 (m/z 1103.47) fragment ions provide a phosphorylation sites on Ser866 in

^{864}SLSVEIVYEGK^{874} peptide The predominant neutral loss ions are observed in y9/y9-98 (m/z 1103.47/1002.60), which suggest that ^{864}SLSVEIVYEGK^{874} is phosphorylated (Figure 4).

To investigate phosphorylated state of TOPORS, His-tagged TOPORS purified from H1299 cell lysates were treated with or without calf intestine phosphatase for 2 hours. Phosphatase treatment caused TOPORS to migrate faster (Figure 5), indicating there are possible phosphorylation sites on TOPORS.

Phosphorylation of Ser98 of TOPORS has been reported previously in screening for nuclear phosphorylated proteins in HeLa cells (Beausoleil et al., 2004; Beausoleil et al., 2006) and is located near the conserved RING domain, which is demonstrated to be required for the ubiquitin ligase activity of TOPORS (Rajendra et al., 2004). Therefore, Ser98 has been selected for further investigation to examine the importance of Ser98 in regulating the ubiquitin ligase activity of TOPORS.

DISCUSSION

As demonstrated previously by others (Wu et al., 2007), alkaline phosphatase treatment to remove phosphate groups produced a mass shift of 80 Da relative to the original masses of the phosphopeptide . The migration shift, observed when TOPORS was expressed in mammalian cells, may be due to post-translational modifications (Figure 1). However, phosphatase treatment did not completely dephosphorylate phosphate groups from TOPORS peptides for LC-MS/MS analysis.

Several predicted phosphorylation sites on TOPORS are located near RING domain, SR dipeptides and SUMO binding motif (SBM) (http://www.cbs.dtu.dk/services/NetPhos/; http://973-proteinweb.ustc.edu.cn/gps/gps_web/). Four phosphorylated residues of TOPORS identified are Ser98, Ser499, Ser585 and Ser866, which are conserved among the TOPORS orthologs (Figure 6). Ser98 is located near the RING domain while Ser499 and Ser866 are located in SUMO-1 interacting motif (SIM). TOPORS is the first protein with both ubiquitin and SUMO E3 ligase activity. The conserved N-terminal RING domain of TOPORS is necessary for ubiquitin E3 ligase activity (Rajendra et al., 2004). Mutations in RING domain (W131A) or TOPORS fragment lacking the RING domain inactivated its activity for p53 ubiquitination, indicating that the RING domain is required for ubiquitination activity (Rajendra et al., 2004). TOPORS also functions as a SUMO-1 E3 ligase for p53. The RING domain is dispensable for SUMOylation activity (Weger et al., 2003). Furthermore, a recent study from our group (Kulkarni, et al.,

submitted) indicates that the SUMOylation activity of TOPORS requires a conserved SUMO-1 binding motif (SBM; residues 478-481; CVIV). Studies of PIAS proteins implicate this SUMO-1 interacting motif (SIM) binds directly to SUMO-1. SIM of PIASxa contains a short motif of hydrophobic amino acids followed by acidic amino acid residues (Minty et al., 2000). Furthermore, phosphorylation of a residue near the SUMO-1 interacting motif (SIM) of PIASxa enhances its binding to SUMO-1 (Hecker et al., 2006). TOPORS also contains SIM (residues 478-509; CVIVGFVKPLAER TPELVELSSDSEDLGSYEK). The corresponding residues in the SIM of TOPORS and PIASxa are highly conserved. Phosphorylated Ser499 is located within SIM. Therefore, it is possible that TOPORS ubiquitin and SUMO E3 ligase activities are regulated by phosphorylation of residues near the RING and SIM domains, respectively.

Although one type of post-translational modifications, phosphorylation, was investigated, it was reported that TOPORS is SUMOylated at Lys 560 (Weger et al., 2003). Because only a small fraction of substrate, often less than 1%, is SUMOylated at any given time, it is possible that the sensitivity of mass spectrometric approach used in this study was insufficient to detect SUMOylation sites on TOPORS (Denison et al., 2005). It is difficult to identify SUMOylation sites in mammalian cells because the C-terminal regions of mammalian SUMO-1,-2 and -3 lacks Lys or Arg residues (Knuesel, 2005). Other TOPORS modifications might have been missed due to incomplete coverage of TOPORS or the genesis of short peptides of only a few amino acids after enzymatic digestion, which are not detectable by typical LC-MS/MS. Treatment of H1299 cell lysates transfected with His-tagged TOPORS, with calf intestine alkaline

phosphatase (CIP) did not result in a significant decrease in the migration of TOPORS on SDS-PAGE gels (Figure 5). It is difficult to indentify ubiquitin modification because the turnover of ubiquitinated proteins is very rapid so that steady-state conjugate levels are characteristically low. Ubiquitin is covalently attached to the target Lys residue via an isopeptide bond. The signature ubiquitin peptide after trypsin digestion has a mass shift of 114.1 Da (Peng et al., 2003).

While investigating posttranslational modification sites on TOPORS, other proteins were identified. All of these proteins have MASCOT scores above 100 and more than 10 tryptic peptides. It is possible that these proteins interact with TOPORS and co-precipitated with TOPORS during nickel based affinity chromatography. Alternatively, it cannot be ruled out that those proteins co-purified as a result of interactions with the affinity matrix rather than TOPORS. Several of these putative TOPORS-interacting proteins are involved in nuclear mRNA processing or chromatin modification (Table 2). In addition, several of them were previously shown as potential SUMOylation substrates for TOPORS(Pungaliya et al., 2007).

Alteration in chromatin structure or regulation was shown to change sensitivity to histone deacetylase inhibitors such as trichostatin A (TSA) (Bandyopadhyay et al., 2004; Ropero et al., 2006). Since many identified substrates for TOPORS were involved in chromatin regulation, *Topors* +/+ and *Topors* -/- MEFs were used to investigate whether loss of TOPORS affected TSA sensitivity. The result indicated that loss of TOPORS confers resistance to TSA (Pungaliya et al., 2007). Recently, our group discovered that TOPORS deficient primary embryonic fibroblasts exhibited aneuploidy (Marshall et al.,

submitted). Studies from human and Drosophila TOPORS orthologs imply that TOPORS involved in the chromatin regulation and genomic stability due to the alteration in pericentric heterochromatin formation (Cimini and Degrassi, 2005). Among putative interacting proteins with TOPORS, Sin3A was identified as a potential TOPORS SUMOylation substrate (Pungaliya et al., 2007). It is reported that alterations in mSin3A function result in chromosomal and genomic instability that accelerate tumorigenesis (Dannenberg et al., 2005). It is possible that TOPORS may affect SUMOylation or ubiquitination of its interacting proteins which may result in the alteration of pericentric heterochromatin formation. TOPORS contains RS dipeptides, a characteristic feature of many RNA processing factors (Wilkinson, 2005). It is possible that TOPORS might be a component of the spliceosomes. Before investigating the role of TOPORS on interacting proteins, further validation analysis is required.

Interestingly, ubiquitin peptides were also identified in the mass spectrometry analysis (Table 3). Although the significance of this finding is unclear, TOPORS and other E3 ubiquitin ligases induce formation of high molecular weight polymeric ubiquitin chains, and it can be speculated that co-purification of these chains with TOPORS might have yielded the ubiquitin peptides.

Section 2.

Investigation of the role of serine 98 phosphorylation in the ubiquitin and SUMO ligase activities of TOPORS

INTRODUCTION

TOPORS was shown to function as both a ubiquitin and SUMO E3 ligase, and is the first protein such dual activities through two distinct domains. A conserved N-terminal RING domain of TOPORS is necessary for the ubiquitin E3 ligase activity (Rajendra et al., 2004). Deletion of RING domain inactivates the ubiquitination activity of TOPORS (Rajendra et al., 2004). In addition, the mutation of Trp131 (W131A), which is a conserved residue among TOPORS orthologs within TOPORS RING domain, inactivated its activity towards p53 ubiquitination (Rajendra et al., 2004). These results indicate that the RING domain is required for ubiquitination activity (Rajendra et al., 2004). The RING domain of TOPORS is highly similar to that of CBL. Structural study of CBL bound to the E2 UbcH7 shows that the CBL RING domain is responsible for E2 binding (Zheng et al., 2000). TOPORS also functions as a SUMO-1 E3 ligase for p53, with the RING domain, which is dispensable for this SUMOylation activity (Weger et al., 2005). Furthermore, a recent study from our group (Kulkarni, et al., submitted) implies that the SUMOylation activity of TOPORS requires the residues containing a SUMO-binding motif (SBM; residues 478-481), which is implicated in binding SUMO orthologs (Weger et al., 2003).

However, there are other E2 and E3 enzymes possessing dual UBL conjugation specificity. One example is the E2 UbcH8, which is reported to possess dual ubiquitin and ISG15 conjugation activity (Zou and Zhang, 2006). MDM2 also functions as a dual

ubiquitin and NEDD8 E3 ligase (Harper, 2004; Xirodimas et al., 2004). A nuclear multi-protein complex involved in DNA repair also possesses dual ubiquitin/SUMO ligase activity. This complex contains the protein MMS21/NSE2 as a SUMO E3 ligase and NSE1 as a RING-type ubiquitin E3 ligase (Potts and Yu, 2005, 2007; Santa Maria et al., 2007).

As described In Section 1 of this thesis, E3 ligase activity can be regulated by phosphorylation. A recent study showed that phosphorylated Y371 affects the CBL-E2 interaction and the ubiquitination activity of CBL (Yokouchi et al., 2001). Y371 of Cbl was shown to be a part of a loop structure that binds the E2 UbcH7 via an alpha helix, which contacts two loop structures of UbcH7. This interface contains primarily polar and charged residues, which create intermolecular hydrogen bonds (Zheng et al., 2000) The majority of the residues within the Cbl helix, as well as the loop structures of UbcH7, are conserved (Eletr and Kuhlman, 2007). Tyr phosphorylation of c-Cbl affects its ability to bind to UbcH7 and enhances its ubiquitination activity by changing its conformation (Kassenbrock and Anderson, 2004; Kassenbrock et al., 2002). Phosphorylated residue of a ubiquitin E3 ligase can disrupt the local stability of the structure (Penrose et al., 2004) and change the local structure that can be recognized as a motif for a specific ubiquitin conjugating enzyme or as a ubiquitin acceptor. An incorporated phosphate group can act as a helix breaker, which disturbs the protein structure and affects its stability (Zetina, 2001). Cbl Y371E mutants, imitating phosphorylated Tyr, are constitutively active (Kassenbrock and Anderson, 2004).

Phosphorylated Y371 makes CBL susceptible to protease digestion, presumably by altering the conformation of CBL (Kassenbrock and Anderson, 2004).

Hypophosphorylation of the E3 ligase Mdm2 augments stability of p53 by lowering its ubiquitination activity (Blattner et al., 2002). In contrast to CBL, in which phosphorylated residue near the RING domain affects an E2 interaction, phosphorylated Ser 186 of MDM2 is not located near the RING domain, but in Akt consensus site (RxRxxS/T). Phosphorylated Ser186 is necessary for its translocation from cytoplasm to nuclear and thus increase its ubiquitination activity on p53. (Ashcroft et al., 2002; Ogawara et al., 2002; Zhou et al., 2001).(Michael and Oren, 2003). However, Tyr phosphorylation by ErbB-4 Tyr kinase on Mdm2 stimulates its own degradation and results in increased p53 levels (Arasada and Carpenter, 2005).

Itch, a HECT E3 ligase, was also found to be directly activated upon phosphorylation on the PRR (Pro-rich region) motif since unphosphorylated PRR inhibits its catalytic activity by engaging in an intramolecular interaction between the HECT and WW domain. Phosphorylation of PRR alters from closed to open conformation that weakens the interaction between HECT and WW domain and thereby increases its catalytic activity (Gallagher et al., 2006).

ICP0, another RING-type E3 ubiquitin ligase (Boutell and Everett, 2004), may also be regulated by phosphorylation. The expression level of a phospho-deficient ICP0 mutants (residues S508, S514, S517, and T518 are replaced to Ala) is decreased when compared to that of the wild type protein (Davido et al., 2005). A phosphomimic mutant (T10E) of SAG (Sensitive to Apoptosis Gene), a RING component of a SCF E3 ubiquitin ligase,

has a much shorter protein half-life than wild type SAG (Avraham et al., 2007). With regard to SUMOylation, phosphorylated T495 in Pc2, a known SUMO E3 ligase, by HIPK2 (homeodomain interacting protein kinase 2) increases its SUMOylation activity, which reveals the regulation of the SUMO E3 ligase activity by its substrate protein (Roscic et al., 2006).

To investigate how phosphorylation might regulate the TOPORS E3 ligase activity, studies were focused on the conserved S98. Phospho-deficient and phosphomimic mutants of Ser98 were generated to allow the analysis of the function of mutation both in cells and *in vitro.*

RESULTS

Analysis of the effects of serine 98 mutations on the ubiquitination activity of TOPORS in cells

Since physiologic substrates of TOPORS are not identified yet, in order to investigate the role of phosphorylation of Ser98, I took advantage of the capability of TOPORS in inducing both polymeric ubiquitin (Rajendra et al., 2004) and polymeric SUMO-1 chains (Hammer et al., 2007) (Kulkarni, et al., submitted) in the absence of a specific substrate is utilized. In HEK293 cells co-transfected with vectors expressing both polyhistidine-tagged ubiquitin and a phosphomimic TOPORS mutant (S98D), there was an increase in steady-state levels of high molecular weight ubiquitin conjugates, relative to cells expressing either wild-type TOPORS or a phospho-deficient TOPORS mutant (S98A) (Figure 7A). Similar results were obtained in cells transfected only with TOPORS vectors (Figure 7B). In both transfection strategies, it has been observed that expression of the S98D TOPORS mutant was lower than that of the wild-type or S98A mutant (Figure 7A and 7B). When cell lysates were equilibrated for relative TOPORS protein expression, an even greater effect of the S98D mutant on high molecular weight ubiquitin conjugates was observed (Figure 7C). To determine whether the S98D mutation affected the protein stability, cells were transfected in the absence or presence of the proteasome inhibitor MG132. Addition of MG132 significantly blocked the degradation of TOPORS phosphomimic mutants (S98D) (Figure 7D). This finding

suggests that the decreased expression level of the phosphomimic mutant (S98D) is due to increased proteasomal degradation.

Analysis of the effects of mutations of serine 98 on the SUMOylation activity of TOPORS in cells

In addition, the role of S98 mutation of S98 affected on the TOPORS SUMO ligase activity was investigated. In cells co-transfected with vectors expressing polyhistidine-tagged SUMO-1 and TOPORS, there was no difference in the levels of high molecular weight SUMO-1 conjugates among cells expressing wild-type, S98D, or S98A TOPORS proteins (Figure 8). Similar results were obtained in cells transfected only with TOPORS vectors (Figure 8). These findings suggest that in contrast to ubiquitination, mutation at Ser98 residue had no effect on the SUMOylation activity of TOPORS. This result is also consistent with previous reports indicating that the TOPORS ubiquitin and SUMO E3 ligase activities involve two distinct domains in the protein: the RING domain and a region containing a SIM-like motif (Hecker et al., 2006; Rajendra et al., 2004)

Mutations of Ser98 of TOPORS did not affect cellular localization.

Since our group previously demonstrated that TOPORS co-localizes with PML nuclear bodies in a dynamic manner (Rasheed et al., 2002), it was investigated whether the alteration of S98 affected on the cellular localization of TOPORS was examined.. Transfection studies with GFP-TOPORS constructs indicated that neither S98D nor S98A mutations affected changed the punctate nuclear localization pattern of TOPORS.

As a negative control, TOPORS RINGless (residues 231-1045) and W131A mutants were used (Figure 9).

Analysis of the effects of mutations of serine 98 on the ubiquitination/SUMOylation activity of TOPORS in vitro

To directly investigate the role of Ser98 in the ubiquitination and SUMOylation activity of TOPORS, *in vitro* assays were performed using purified components (E1 and E2 enzymes) and GST-TOPORS fusions proteins expressed in bacteria. Previous studies indicated that under these conditions, TOPORS was capable of stimulating formation of polymeric ubiquitin (Rajendra et al., 2004) and SUMO conjugates (Kulkarni et al., manuscript submitted) in the absence of specific substrates. Furthermore, both ubiquitin and SUMO conjugates were detectable in experiments in which both the ubiquitination and SUMOylation reaction components were combined (Kulkarni et al., manuscript submitted). These findings allowed us to quantify the ubiquitination and SUMOylation activity of the S98 mutants in a single tube assay. To explore the kinetics of this assay, the ubiquitin chain formation was examined at several early time points (0, 15, 30 minutes) for the wild-type and RINGless TOPORS enzymes (residues 231-1045). As a negative control, RINGless TOPORS was used since it was inactive in the ubiquitination activity (Rajendra et al., 2004). The results showed that the ubiquitin chain formation continued to increase up to 30 minutes (Figure 10).

When a dual ubiquitination and SUMOylation reaction was performed for 15 minutes, a greater accumulation of high molecular weight ubiquitin conjugates was observed with

the S98D mutant when compared to wild-type or the S98A mutant (Figure 11). Importantly, mutation of S98 had no effect on the SUMOylation activity of TOPORS (Figure 11), excluding a small change in protein content that might have confounded the results of ubiquitination assay. Thus, consistent with the cellular transfection data, a phosphomimic mutation of S98 conferred an increase in the ubiquitination but not SUMOylation activity of TOPORS.

Mutation of S98 of TOPORS affects binding to the E2 enzyme UbcH5a

RING domains of E3 ligases were shown to bind E2 enzymes, with specific residues both within and adjacent to the RING domain involved in this binding (Freemont, 2000; Joazeiro and Weissman, 2000). In addition, a structural study of the CBL-UbcH7 complex suggested that phosphorylation of residue Tyr371 on c-Cbl increased its ubiquitination activity by affecting E2 binding (Levkowitz et al., 1999) (Yokouchi et al., 2001; Zheng et al., 2000). Previously, our group reported that TOPORS stimulated p53 ubiquitination with selected E2 enzymes (UbcH5a, UbcH5c, and UbcH6 but not UbcH7, CDC34, and UbcH2a) (Rajendra et al., 2004). Thus, it was investigated whether the S98 was involved in E2 binding, using an in vitro pull-down assay with purified GST-TOPORS proteins and UbcH5a.

Minimal binding of UbcH5a to wild-type TOPORS was observed (Figure 12). Similarly, minimal, if any, UbcH5a binding was detected using the S98A mutant (Figure 12). By contrast, binding of UbcH5a to the phosphomimic mutant (S98D) was easily detectable (Figure 12). This result provides an explanation for the increased ubiquitin

ligase activity observed with the S98D mutant in cells and *in vitro*, and suggests that S98 is involved in the binding of UbcH5a to TOPORS.

DISCUSSION

The possible functional role of Ser98 in TOPORS

Results from *in vivo* and *in vitro* assays indicated that a phosphomimic S98D mutant of TOPORS was more active in ubiquitination than wild type or a phospho-deficient mutant. However, the S98D mutant did not affect the SUMOylation activity of TOPORS in cells or *in vitro*. These results suggests that Ser98 may serve as a molecular switch;: when Ser98 is phosphorylated, TOPORS may prefer ubiquitination over SUMOylation. It is reported that longer ubiquitin chains can be assembled on E2 enzyme before being transferred to substrates (Brzovic et al., 2006; Li et al., 2007). Even though several publications indicated potential substrate for TOPORS (Table 4), physiological relevant substrates and the biological role of TOPORS is poorly understood. A proteomic screen of candidate SUMOylation substrates for TOPORS indicated that many of them are involved in the chromatin modification or the transcriptional regulation (Pungaliya et al., 2007). Since many of them appeared during the previous His-TOPORS protein purification, it may be possible that they might be ubiquitination or SUMOylation substrates for TOPORS. There is an emerging evidence that certain ubiquitin E3 ligases target SUMOylated substrates via SIMs (Berta et al., 2007) (Mullen and Brill, 2008). Since TOPORS has several SIMs (residues 478-509 and 906-916), it is possible that TOPORS may function as an E3 ligase for poly-SUMOylated substrates, with this activity regulated by S98 phosphorylation. Additional studies are needed to determine

whether phosphorylation of S98 in TOPORS affects the ubiquitination or SUMOylation activity on TOPORS towards specific substrates. Notably, our laboratory observed the alterations in the localization of HP1α (heterochromatin protein α), required for proper chromosomal segregation (Hiragami and Festenstein, 2005) in TOPORS-deficient MEFs (Henderson et al, manuscript submitted). These findings suggest that one cellular role of TOPORS may be involved in the regulation of centromeric substrates, perhaps by ubiquiting them.

TOPORS contains five PEST sequences, which are rich in SP/TP motifs. These motifs are a minimal recognition element for Ser/Thr kinases. PEST sequences are involved in 26S proteasome dependent protein degradation (Rechsteiner and Rogers, 1996). It was reported that short-lived proteins contains PEST sequence which are rich in Pro, Glu, Ser, or Thr (Rogers et al., 1986). The expression levels of short-lived proteins such as transactivators Gcn4 or G1 cyclins is controlled via PEST sequences (Kornitzer et al., 1994). Phosphorylation of a particular Ser or Thr within the PEST sequence of G1 cyclins specifies their recognition and degradation processing by the 26S proteasome dependent pathway (Willems et al., 1996; Won and Reed, 1996). NMR and circular dichroism data revealed that phosphorylation of the PEST region of viral E2 proteins destabilizes the structure of these peptides by changing the conformation, and leading to increased degradation of the E2 protein within the cell (Garcia-Alai et al., 2006). Phosphorylated Ser98 is found in a tryptic digested ^{89}LQQTVPADASPDSK^{102} peptide. This peptide contains PEST like sequence (residues 99-102) and a predicted loop structure. The phosphomimic S98D TOPORS mutant showed a rapid turnover compared

to wild type and phospho-deficient TOPORS mutant (S98A). Therefore, analogous to other PEST-containing proteins, phosphorylation at Ser98 might confer rapid degradation. Additionally, phosphorylated S499 is located in another PEST sequence (residues 490-509) (Figure 13).

Regulation of the ubiquitination activity of CBL by phosphorylation

c-CBL ubiquitinates the activated PDGF, EGF and CSF-1 receptor Tyr kinases (RTKs), resulting in attenuating signaling pathways. These cellular signaling pathways are involved in DNA synthesis, cell proliferation, and cytoskeletal reorganization. Point mutagenesis and crystal structure studies clarified the interaction between CBL and UbcH7. C-CBL binds UbcH7 through its RING domain and its linker helix. The RING domain of c-CBL provides a groove formed by an alpha helix and two zinc-chelating loops (loop1 and loop2) (Figure 17). This groove contacts with L1 loop and L2 loop of UbcH7 which creates multiple van der Waals interactions. However, the linker helix of c-CBL and the H1 helix of UbcH7 contain primarily polar and charged residues, which form intermolecular hydrogen bond contacts. In addition, the structure of c-CBL indicates that phosphorylation of Tyr371 within the linker helix would result in a conformational change in the linker helix of c-CBL. This change would make Tyr371 solvent accessible and alter the UbcH7 interaction (Zheng et al., 2000).

Phosphorylation of Tyr 371 of c-CBL plays an important role in the E3 ligase activity of c-CBL (Levkowitz et al., 1999). Kassenbrock and Anderson et al. reported that c-CBL containing a Tyr to Glu substitution of Y371 exhibited constitutive E3 activity

towards EGFR (Kassenbrock and Anderson, 2004). Phosphorylation of Tyr 371 is also important for c-CBL-dependent ubiquitination of Src *in vitro* (Yokouchi et al., 2001). Y371F and Y368F/Y371F mutants were inactive in autoubiquitination activity in vitro, while Y371E and Y368E/Y371E mutants were constitutively active and did not require Tyr phosphorylation for their E3 ligase activity (Kassenbrock and Anderson, 2004). Tyr 371 phosphorylation may also affect the release of the activated ubiquitin from the E2 enzyme (Yokouchi et al., 2001).

Mechanism of ubiquitination activity of TOPORS

Protein-protein interaction could be regulated by phosphorylation. Many publications indicate that RING-type E3 ligases bring the target protein to the activated ubiquitin-E2 and help to orient the substrate for optimal ubiquitin conjugation (Cheng and Hart, 2001; Kaiser and Tagwerker, 2005; Vosseller et al., 2001). Furthermore, RING domain of E3 ligases can interact specifically with E2 enzymes which interaction is required for ubiquitinating target proteins (Freemont, 2000; Joazeiro and Weissman, 2000). It has been reported that RING type E3 ligase stimulates the rate of ubiquitin release of E2-ubiquitin thiolesters which results in the autoubiquitination of E2 enzymes or the synthesis of polyubiquitin chains in the absence of substrates (Furukawa et al., 2002; Gmachl et al., 2000; Joazeiro et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tang et al., 2001). When UbcH5a, an E2 enzyme, binds to the E3 ligase, it results in the subtle conformational change of I37 residue in UbcH5a. This finding supports the notion that RING type E3 ligases activate E2 enzymes in an allosteric fashion and enhance

ubiquitination on the target proteins (Ozkan et al., 2005). RING domains of E3 ligases such as c-CBL were shown to interact with E2 enzyme (Zheng et al., 2000). The sequence of RING domain of TOPORS is similar to that of CBL (Figure 14). Prediction algorithms (www.predictprotein.org) suggest that Ser98 of TOPORS is also part of a loop (Figure 14). Therefore, it can be speculated that similar to Cbl, S98 of TOPORS is part of a loop structure that is important in E2 binding. If S98 interacts with polar or charged residues of E2 enzymes such as UbcH5a, phosphorylation of S98 may significantly increase this interaction, and thus regulate E2 binding by TOPORS.

Predicted regulation of the SUMOylation activity of TOPORS

It was demonstrated that phosphorylation near the SUMO interacting motif of PIASxa (SUMO E3 ligase) increased its binding affinity to SUMO-1 (Hecker et al., 2006). The SUMO interacting motif contains V/I-X-V/I-V/I residues and acidic amino acids. Phosphorylation of Ser as well as negative charged acidic residues within the PIAS SIM help to maintain specificity and orientation of binding of SUMO1 by increasing the affinity through additional electrostatic interactions. Deletion of an acidic amino acid stretch of TOPORS reduced SUMO-1 binding, whereas SUMO 2/3-binding was not altered (Hecker et al., 2006). Ser499 of TOPORS is located near a conserved SUMO interacting motif (residues 478-509). Thus, phosphorylated Ser499 could stabilize an interaction between TOPORS and SUMO-1.

RanBP2 can form polymeric SUMO chains through its core catalytic domain IR1-M-IR2, which contains a SIM-like motif and is implicated in binding both SUMO-1 and

Ubc9 (Pichler et al., 2002). In addition to the residues 478-481 of TOPORS, two conserved sequences of TOPORS (residues 315-318 (VNIV) and residues 876-870 (VEIV)) are similar to SUMO-interacting motifs. Weger et al. mapped a SUMO-binding regions of TOPORS to residues 415-684 and residues 911-917 using yeast two-hybrid assays. Residues 911-917 of TOPORS were also mapped as a Ubc9-binding region (Weger et al., 2003).

Regulation of TOPORS E3 ligase activity by extracellular signal

Additional studies are needed to identify the kinase(s) responsible for phosphorylation of TOPORS. The regulation of other E3-substrate interactions occurs through phosphorylation of either the substrate or the E3 ligase. For example, IkB kinase (IKK) phosphorylates IkBa, leading to ubiquitination and degradation. Stress-induced PI3K family members, such as ATM, ATR, and DNA-PK, may regulate Mdm2 by enhancing autoubiquitination and proteasome-mediated degradation in response to DNA damage (Stommel and Wahl, 2004). The E3 ligase Itch is phosphorylated by c-Jun NH_2-terminal kinase-1 after T cell receptor engagement, which enhances its enzymatic activity (Gallagher et al., 2006). However, phosphorylation of Itch by Fyn inactivates its ubiquitin E3 ligase activity (Yang et al., 2006). p38, a MAPK activated by cytokines, reactive oxygen species, and DNA damage as well as hypoxia phosphorylates Siah2 (Khurana et al., 2006). Activated Siah2 increases its ubiquitination activity towards PHD3 (Khurana et al., 2006). Nedd4-2, an E3 ligase, was found to be phosphorylated by

serum glucocorticoid-inducible protein kinase 1 (SGK-1), which increased its ubiquitination activity and thus induced SGK degradation. (Zhou and Snyder, 2005).

In a transcriptional profile study of TOPORS primary +/+ and -/- MEF cells performed by our laboratory, transcripts of 3 Wnt-related genes (secreted frizzled related sequence protein 2(Sfrp2), leucine rich repeat containing G protein coupled receptor 5 (Lgr5), WNT1 inducible signaling pathway protein 2 (Wisp2)) were found to be upregulated in TOPORS -/-MEF cells (Henderson et al, manuscript submitted). This result suggests that TOPORS may affect Wnt signaling pathway or vice versa. It is reported that several E3 ligases are involved in regulating Wnt signaling pathway. NARF (NLK associated RING finger protein) induces the ubiquitination of TCF/LEF (transcription factor T-cell factor /lymphoid enhancer factor 1), a repressor of Wnt target genes that subsequently degraded by the proteasome. This negatively regulates the Wnt signaling pathway (Yamada et al., 2006). Siah1, a known ubiquitin E3 ligase, promotes β-catenin degradation, resulting in inhibition of the Wnt/β -catenin pathway (Park et al., 2003). Cullin4B/E3-ubiquitin ligase downregulates β-catenin (Tripathi et al., 2007). PIASy sumoylates lymphoid enhancer factor 1 (LEF1), and represses its activity, which is part of the Wnt signaling pathway (Sachdev et al., 2001). It is possible that TOPORS may regulate Wnt signaling pathway by ubiquitinating or sumoylating proteins involved in this pathway. Conversely, members of the Wnt pathway might phosphorylate TOPORS that can regulate its enzymatic activity.

There are several lines of evidence implicating cross-regulation between protein ubiquitination/ UBL modification and protein phosphorylation events. Phosphorylated c-

CBL promotes ubiquitination of itself and turnover of Tyr kinase, Src. This negatively regulates Src-dependent phosphorylation of c-CBL at a conserved Tyr, implicating the existence of a feedback loop between the target protein and its E3 ligase (Yokouchi et al., 2001). HIPK2 induced by DNA damage phosphorylates Pc2, a SUMO E3 ligase, Phosphorylated Pc2 enhanced its SUMOylation activity towards HIPK2, resulting in transcriptional repression presumably by mechanisms that employ interactions with corepressors such as Groucho (Sung et al., 2005) or coactivators including the binding partner CBP (Hofmann et al., 2002; Roscic et al., 2006). S90 phosphorylation of PIAS regulates its SUMO E3 ligase activity and is required for repressing transcription (Liu et al., 2007).

A phosphorylation level of <10% of the corresponding unphosphorylated peptides (residues 89-102) was estimated based on relative peak intensities as observed by LC-MS/MS analyses of TOPORS peptides (Figure 19). TOPORS activity might be induced by kinase(s) which may phosphorylate S98. ^{89}LQQTVPADA**S**PDSK^{102} contains a mitogen-activated protein kinase (MAPK) phosphorylation site and a SP motif (Pro-directed Ser/Thr) (Figure 15), which is conserved among TOPORS orthologs. (Gallagher et al., 2006).

Other predicted kinases for Ser98 of TOPORS include the CMGC group (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinases (GSKs) and CDK-like kinases (CLKs)) (Kannan and Neuwald, 2004) (http://pred.ngri.re.kr/PredPhospho.htm) (http://www.cbs.dtu.dk/services/NetPhos/;

http://973-proteinweb.ustc.edu.cn/gps/gps_web/). Additionally CK2 is a predicted kinase for S499 of TOPORS (Table 5).

SUMMARY OF THE DISSERTATION

TOPORS was originally identified as a topoisomerase I and p53 interacting protein. It contains a conserved C3HC4 RING domain at the N-terminus that is required for ubiquitin E3 ligase activity towards p53 and the Hairy transcriptional repressor. Expression and genetic studies suggests that TOPORS may be a tumor suppressor in colon, lung and brain malignancies (Bredel et al., 2005; Lin et al., 2005; Saleem et al., 2004). The RING domain of TOPORS shares sequence similarity with CBL and ICP0, which are RING-dependent ubiquitin E3 ligases. By contrast, RING domain is dispensable for SUMO E3 ligase activity, which maps to residues 437 to 574 (Weger et al., 2003). Post-translational modifications of E3 ligases have been shown to regulate their enzymatic activities. In particular, phosphorylation of a ubiquitin or SUMO E3 ligase may influence its activity by affecting E2 enzyme interaction, substrate binding, cellular localization, or protein stability (Zheng et al., 2000) (Winkler et al., 2004).\ (Swaminathan and Tsygankov, 2006) (Kassenbrock et al., 2002) (He et al., 2007; Hecker et al., 2006; Khurana et al., 2006).

Potential phosphorylation sites of S98, S499, S585, and S866 are identified (Table 1). Among them, serine 98 was chosen for the project, since several lines of evidence suggested that this residue might be important in the ubiquitination activity of TOPORS.

Since physiological substrate for TOPORS are not yet known, we take advantage of the finding that TOPORS induces both high mobility ubiquitin and SUMO chain

formation in the absence of specific substrates (Rajendra et al., 2004). The cellular study indicates that cells expressing a phosphomimic mutant (S98D) shows an increase in high molecular weight ubiquitin chains, compared to cells expressing wild-type TOPORS or a phospho-deficient mutant (S98A) (Figure 7). However, mutation of S98 did not affect induction of high-molecular weight SUMO conjugates by TOPORS in cells (Figure8). Similar results were observed in *in vitro* dual ubiquitin/ SUMO assays. The studies of UbcH5a binding to TOPORS mutants supports the findings from in cell and *in vitro* assays by demonstrating that UbcH5a binding is increased in the S98D mutant (Figure 12).

In summary, Ser98 is identified as a phosphorylated residue in TOPORS that induces ubiquitination activity, presumably by increasing E2 binding affinity. Since mutation of Ser98 does not affect the SUMOylation activity of TOPORS, phosphorylation of Ser98 may function as a switch to control ubiquitination versus SUMOylation of TOPORS substrates (Figure 16).

Table 1. Phosphorylated TOPORS peptides detected by LC-MS/MS

(A)

Score[a]	Observed[b]	Mr(expt)[c]	Mr(calc)[d]	Delta[d]	Peptide	Position
85	1089.42	2176.83	2176.93	-0.10	^{491}TPELVELSSDSEDLGSYEK^{509} + Phospho (ST)	499
64	652.22	1302.43	1302.61	-0.18	^{864}SLSVEIVYEGK^{874} + Phospho (ST)	866
64	768.76	1535.51	1535.69	-0.18	^{89}LQQTVPADASPDSK^{102} + Phospho (ST)	98
44	722.25	1442.50	1442.61	-0.11	^{580}GDRVYSPYNHR^{590} + Phospho (ST)	585

*An individual peptide was considered as a good match if it produced a probability-based MOWSE (MOlecular Weight SEarch) score greater than 30 (P<0.001) (Pappin et al., 1993).

(B)

Peptide	Observed[a]	Mr(theoretical)[b]	Mr(observed)[c]	Delta[d]
^{864}SLpSVEIVYEGK^{874}	652.221	1302.4274	1302.6108	-0.1833
^{491}TPELVELSpSDSEDLGSYEK^{509}	1089.423	2176.8314	2176.93	-0.0985
^{89}LQQTVPADApSPDSK^{102}	768.7625	1535.5104	1535.6868	-0.1764
^{583}VYpSPYNHR^{590}	558.202	1114.3894	1114.4597	-0.0702

Peptide	Observed[a]	Mr(theoretical)[b]	Mr(observed)[c]	Delta[d]
^{864}SLSVEIVYEGK^{874}	612.2595	1222.5044	1222.6444	-0.14
^{491}TPELVELSSDSEDLGSYEK^{509}	1049.419	2096.8234	2096.9637	-0.1402
^{89}LQQTVPADASPDSK^{102}	728.8205	1455.6264	1455.7205	-0.0941
^{583}VYSPYNHR^{590}	518.1995	1034.3844	1034.4933	-0.1089

*Four phosphopeptides with four phosphorylated residues of TOPORS were identified. Both phosphorylated and the corresponding non-phosphorylated peptide were detected in H1299 cells (upper panel), whereas in bacteria (lower panel), only non-phosphorylated peptides were identified.

[a]Observed, measured m/z value for the peptide

[b]Mr (expt), peptide mass calculated from observed m/z value

[c]Mr (calc), identified TOPORS peptide mass resulting from *in silico* enzyme digested TOPORS sequence

[d]Delta, the mass difference between the measured and calculated peptide mass

Table 2. Proteins co-purified with TOPORS

gi|862330	DRPLA [Homo sapiens]
gi|7661936	scaffold attachment factor B2 [Homo sapiens]
gi|7512295	atrophin-1 - human
gi|7022183	unnamed protein product [Homo sapiens]
gi|58257658	KIAA0553 protein [Homo sapiens]
gi|57999481	hypothetical protein [Homo sapiens]
gi|54792138	helicase with zinc finger domain [Homo sapiens]
gi|51094661	hypothetical protein MGC20460 [Homo sapiens]
gi|47077085	unnamed protein product [Homo sapiens]
gi|4557449	chromodomain helicase DNA binding protein 2 [Homo sapiens]
gi|4557447	chromodomain helicase DNA binding protein 1 [Homo sapiens]
gi|4507613	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase [Homo sapiens]
gi|435476	cytokeratin 9 [Homo sapiens]
gi|42734323	KIAA0913 [Homo sapiens]
gi|40788952	KIAA0034 [Homo sapiens]
gi|40788877	KIAA0139 [Homo sapiens]
gi|40217847	activating signal cointegrator 1 complex subunit 3-like 1 [Homo sapiens]
gi|40018648	adaptor-related protein complex 3, delta 1 subunit [Homo sapiens]
gi|3901030	cytokeratin type II [Homo sapiens]
gi|38787935	BMP-2 inducible kinase isoform a [Homo sapiens]
gi|386854	type II keratin subunit protein
gi|37540872	PREDICTED: zinc finger protein 609 [Homo sapiens]
gi|3043596	KIAA0536 protein [Homo sapiens]
gi|30379	cytokeratin 17 [Homo sapiens]
gi|30353925	CLTC protein [Homo sapiens]
gi|2827282	TATA binding protein associated factor [Homo sapiens]
gi|27804346	BRD4-NUT fusion oncoprotein [Homo sapiens]
gi|2739087	cofactor of initiator function [Homo sapiens]
gi|2463577	PRP8 protein [Homo sapiens]
gi|24460121	JNK-associated leucine-zipper protein [Homo sapiens]
gi|23397666	transcriptional co-repressor Sin3A [Homo sapiens]
gi|23271902	BMP-2 inducible kinase, isoform b [Homo sapiens]
gi|23271009	Serine/threonine-protein kinase PRP4K [Homo sapiens]
gi|22760651	unnamed protein product [Homo sapiens]
gi|22001417	gemin 5 [Homo sapiens]
gi|21359822	nuclear RNase III Drosha [Homo sapiens]
gi|21237808	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2 isoform b [Homo sapiens
gi|20330554	PRP4 kinase [Homo sapiens]
gi|20140764	WD-repeat protein 33 (WD-repeat protein WDC146)
gi|20139357	Ribonuclease III (RNase III) (Drosha) (p241)
gi|19718731	bromodomain-containing protein 4 isoform long [Homo sapiens]
gi|18676480	FLJ00137 protein [Homo sapiens]

gi|181402	epidermal cytokeratin 2 [Homo sapiens]
gi|17999537	U5 snRNP-specific protein [Homo sapiens]
gi|1770390	SCA2 [Homo sapiens]
gi|1732444	DRPLA protein [Homo sapiens]
gi|1679684	ataxin-2 [Homo sapiens]
gi|1549241	SWI/SNF complex 170 KDa subunit [Homo sapiens]
gi|15422161	transcriptional co-repressor Sin3A [Homo sapiens]
gi|14133229	KIAA0999 protein [Homo sapiens]
gi|1335854	muscle clathrin heavy chain
gi|1177024	Potential helicase with zinc-finger domain
gi|11360158	hypothetical protein DKFZp434P0116.1 - human (fragments)
IPR000626	Ubiquitin
IPR013499	DNA topoisomerase I, C-terminal, eukaryotic-type

*Proteins highlighted in yellow were identified as putative SUMOylation substrates for TOPORS (Pungaliya et al., 2007).

* The listed proteins were MASCOT score above 100 and had at least more that 10 peptides

Table 3. Ubiquitin peptides detected by LC-MS/MS.

Peptide	Observed [a]	Mr(expt) [b]	Mr(calc) [c]	Delta [d]	Score
K.TITLEVEPSDTIENVK.A	894.3785	1786.7424	1786.9200	-0.1776	70
K.IQDKEGIPPDQQR.L	762.3380	1522.6614	1522.7740	-0.1125	68
K.IQDKEGIPPDQQR.L	762.3620	1522.7094	1522.7740	-0.0645	56
K.ESTLHLVLR.L	534.2795	1066.5444	1066.6135	-0.0690	52
R.TLSDYNIQK.E	541.2205	1080.4264	1080.5451	-0.1187	51
K.ESTLHLVLR.L	534.2820	1066.5494	1066.6135	-0.0640	48
R.TLSDYNIQK.E	541.2200	1080.4254	1080.5451	-0.1197	46
K.TITLEVEPSDTIENVK.A	894.3590	1786.7034	1786.9200	-0.2166	42
K.IQDKEGIPPDQQR.L	508.5583	1522.6532	1522.7740	-0.1208	30
R.LIFAGK.Q	324.6610	647.3074	647.4006	-0.0932	30
K.IQDKEGIPPDQQR.L	508.5593	1522.6562	1522.7740	-0.1178	21
K.IQDKEGIPPDQQR.L	508.8610	1523.5612	1522.7740	0.7872	15
K.ESTLHLVLR.L	356.5180	1066.5322	1066.6135	-0.0813	9

[a]Observed, measured m/z value for the peptide

[b]Mr (expt), peptide mass calculated from observed m/z value

[c]Mr (calc), identified TOPORS peptide mass resulting from in silico enzyme digested TOPORS sequence

[d]Delta, the mass difference between the measured and calculated peptide mass

Table 4. Potential substrates for TOPORS

Potential substrates	The role of TOPORS	Reference
P53	Interacts with TOPORS; Targets for ubiquitination; Targets for SUMOylation	(Zhou et al., 1999); (Rajendra et al., 2004); (Shinbo et al., 2005), (Weger et al., 2005)
Nkx3.1	Targets for ubiquitination	(Guan et al., 2008)
Hairy protein (Drosophila)	Mediates polyubiquitination; leads to hairy degradation	(Secombe and Parkhurst, 2004)
Sin3A	Targets for SUMOylation	(Pungaliya et al., 2007)
Topoisomerase I	Targets for SUMOylation	(Hammer et al., 2007)
Mod(mdg4)2.2 and CP190 (chromatin insulator protein) (Drosophila)	Regulate SUMOylation of chromatin insulator proteins	(Capelson and Corces, 2005)
Mx1	Interacting with Mx1 that is associated with PML nuclear bodies	(Engelhardt et al., 2001)

Table 5. Predicted kinases for phosphorylation sites of TOPORS

phosphorylation site prediction servers	position	kinase	Sequence
GPS; PredPhospho	98	CDKs	ADASPDS
GPS	98	CK1	ADASPDS
GPS	98	CK2	ADASPDS
GPS	98	DNA-PK	ADASPDS
GPS	98	GRK	ADASPDS
GPS	98	IKK	ADASPDS
GPS	98	IPL1(yeast)	ADASPDS
GPS	98	KIS	ADASPDS
GPS; PredPhospho	98	MAPK	ADASPDS
GPS; PredPhospho	98	MAPKAPK2	ADASPDS
GPS	98	P34CDC2	ADASPDS
GPS	499	CaM-II	ELSSDSE
GPS	499	CK1	ELSSDSE
GPS; PredPhospho	499	CK2	ELSSDSE
GPS	499	DNA-PK	ELSSDSE
GPS	499	GRK	ELSSDSE
GPS	499	IKK	ELSSDSE
GPS	585	CDKs	RVYSPYN
GPS	585	MAPK	RVYSPYN
GPS	585	P34CDC2	RVYSPYN
GPS	585	PKR	RVYSPYN
GPS	866	AMPK	RSLSVEI
GPS	866	CaM-II	RSLSVEI
GPS	866	CK1	RSLSVEI
GPS	866	MAPKAPK2	RSLSVEI
GPS	866	PAK	RSLSVEI
GPS	866	PHK	RSLSVEI
GPS	866	PKA	RSLSVEI
GPS	866	PKB	RSLSVEI
GPS	866	PKC	RSLSVEI
GPS	866	SGK	RSLSVEI

*Kinases for each phosphorylated residue were predicted by searching database http://pred.ngri.re.kr/PredPhospho.htm, http://www.cbs.dtu.dk/services/NetPhos/; http://973-proteinweb.ustc.edu.cn/gps/gps_web/.

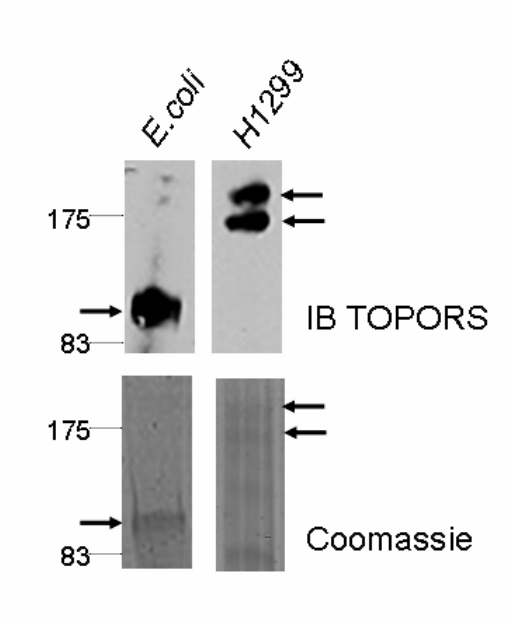

Figure 1. Expression and purification of TOPORS from bacteria and H1299 cells. His-tagged TOPORS was overexpressed in bacteria and H1299 cells, then purified by nickel-based affinity chromatography. A representative immunoblot and Coomassie blue-stained gel are shown. Arrows indicate bands that were excised for mass spectrometry analysis.

Figure 2. Illustration of TOPORS sequence coverage by LC-MS/MS analysis of multiple enzyme digestion including trypsin, Glu-C and Asp-N. MS/MS spectra files (DTA file) were searched against MASCOT search engine. Letters in blue color represent peptides from tryptic digestion (cut K-X or R-X; X is any amino acid). Yellow and green boxes represent peptides produced after Glu-C (cleaves D-X or E-X; X, any amino acid) and Asp-N (cleave X-D and X-E; X, any amino acid) digestion, respectively. Some of peptides from enzyme digestion are overlapping. Total protein coverages is approximately 60% after combining peptides produced after trypsin, Glu-C and Asp-N digestion.

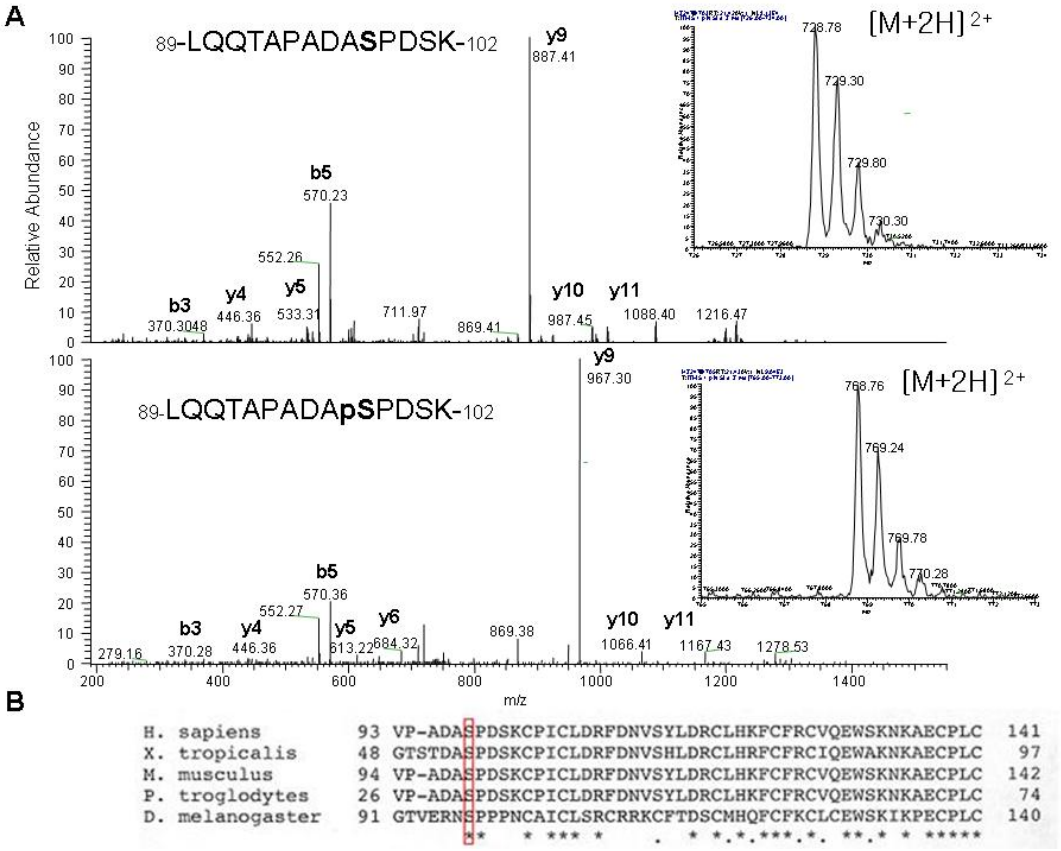

Figure 3. Identification of Ser98 as a phosphorylation site in TOPORS by LC-MS/MS. (A) Zoom scan of double-charged parent ions of both a putative non-phosphopeptide (**^{89}LQQTVPADASPDSK^{102}**), $[M+2H]^{2+}$ at m/z 729.21 and MS/MS spectrum of m/z 729.21 daughter ion from the non-phosphorylated peptide (upper panel). Zoom scan of double-charged parent ions of the corresponding phosphorylated peptide (**^{89}LQQTVPADApSPDSK^{102}**) $[M+2H]^{2+}$ at m/z 768.86 and MS/MS spectrum of m/z 768.86 daughter ion from the phosphorylated peptide (lower panel).

b and y ion refer to ions containing the N- or C-terminal end of the peptide, respectively. Note pairs of y5, Y6, y10, y11 ion peaks from non-phosphorylated and corresponding phosphorylated peptide ions that differ in mass by 80 Da. Instead, y4 ions

are identical for non-phosphorylated and phosphorylated peptide ions. The presence of y4 and y5 ions localizes the site of phosphorylation to S98. (B) Serine 98 in TOPORS is conserved among species. *H. sapiens, X. tropicalis, M.musculus, P. troglodytes, D. melanogaster* TOPORS orthologs were aligned using a ClustalW algorithm. Asterisks indicate identical amino acids. The box indicates the conserved Ser98.

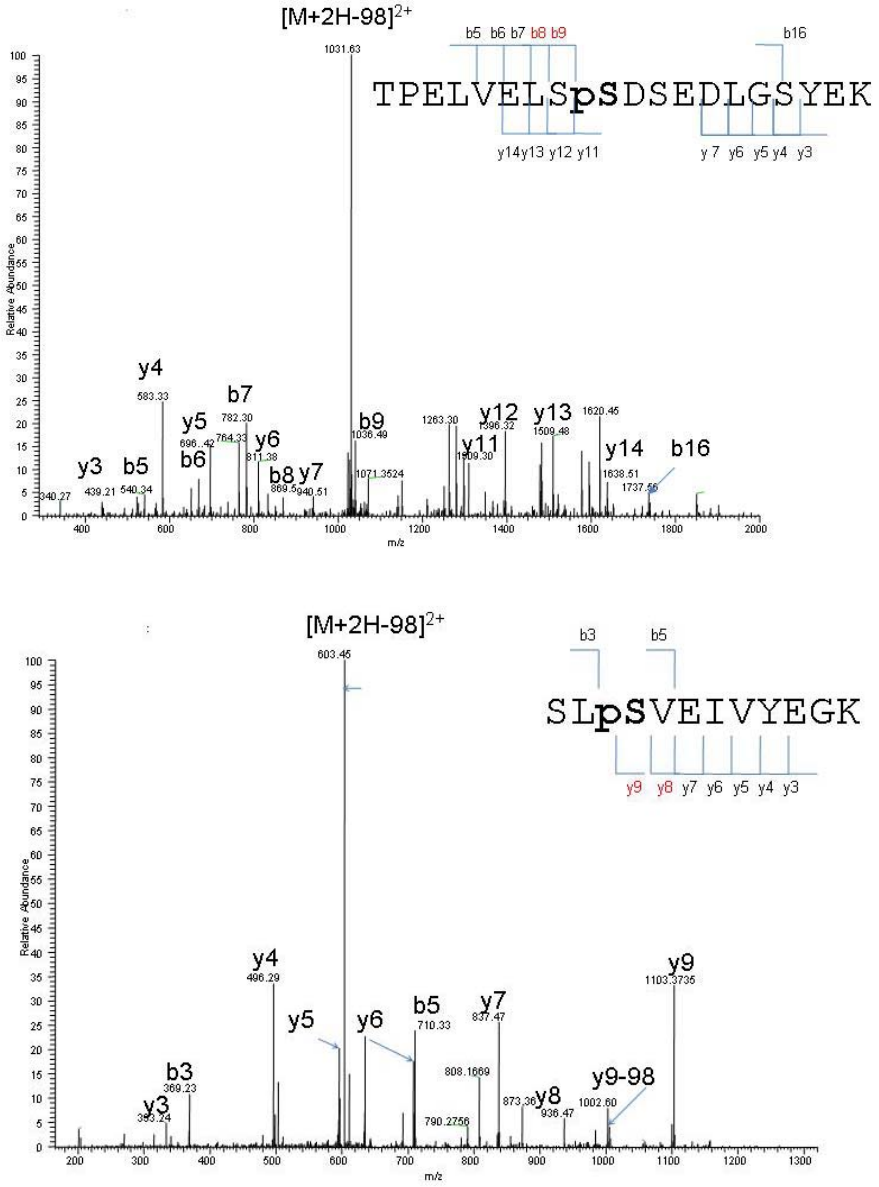

Figure 4. Identification of Ser499 and Ser866 as a phosphorylation sites in TOPORS by LC-MS/MS. (A) monophosphorylated peptide ^{491}TPELVELS**pS**DSEDLGSYEK^{509}. The mass difference between b8 (m/z 833.34) and b9 (m/z 1036.49) ions represents phospho-Ser indicating that S499 is phosphorylated.

(B) MSMSM spectrum of monophosphorylated peptide ^{864}SL**pS**VEIVYEGK^{874}. The prominent neutral loss (-98) ion (603.45) is common phenomenon for peptides that are phosphorylated at Ser or Thr. the presence of y8 (m/z 936.47), y9 (m/z 1103.47) localizes a phosphorylation site on Ser866. Several y ions featured loss of 98 Da due to the loss of phosphoric acid.

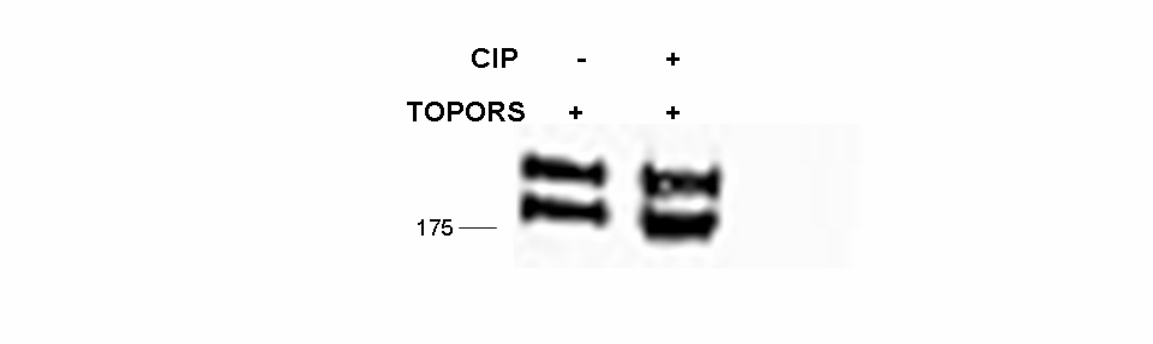

Figure 5. Effects of phosphatase treatment on migration of His-tagged TOPORS in SDS-polyacrylamide gel electrophoresis. Comparison of purified overexpressed His-tagged TOPORS from H1299 cells in the absence and presence of treatment with calf intestine alkaline phosphatase (CIP)

Figure 6. Ser 98, Ser499, Ser585, and Ser866 in TOPORS are conserved among species. *H. sapiens, X. tropicalis, M.musculus, P. troglodytes, D. melanogaster* TOPORS orthologs were aligned using a ClustalW algorithm. Asterisks indicate identical amino acids. The box indicates the conserved Ser98, S499, Ser585 and Ser866.

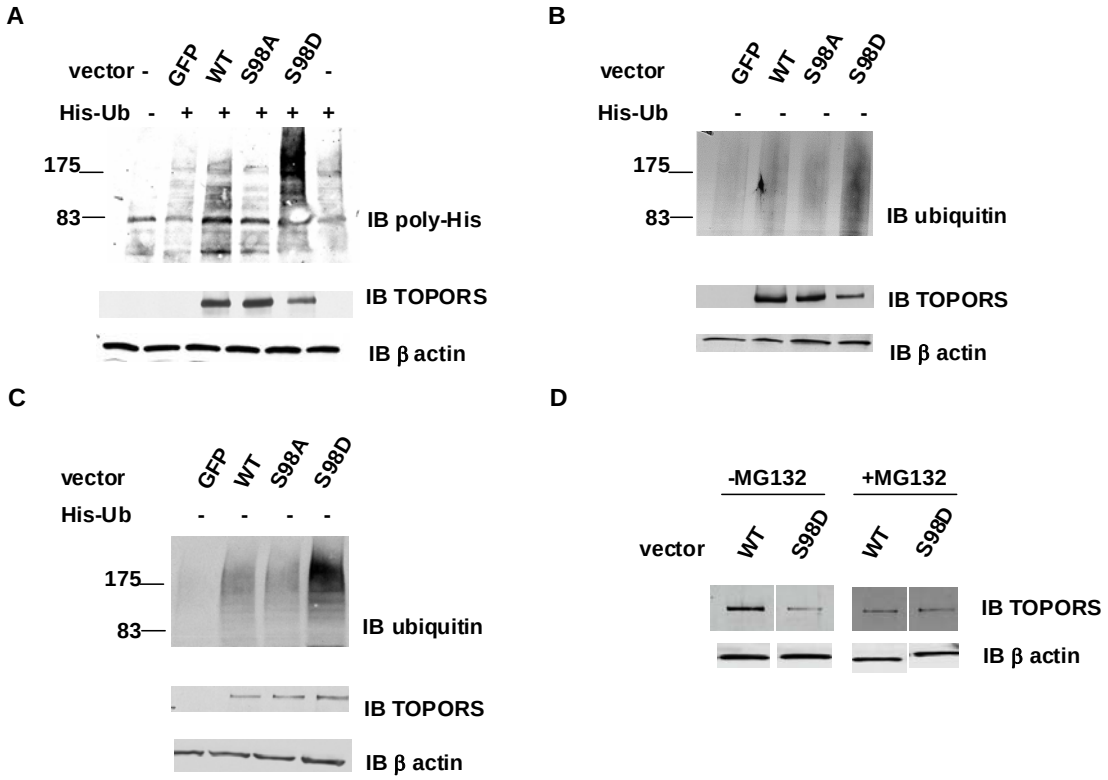

Figure 7. Effects of phospho-deficient (S98A) and phosphomimic (S98D) mutants on the ubiquitination activity of TOPORS in cells. (A) HEK293 cells were transiently transfected with 500ng of a plasmid expressing polyhistidine-tagged ubiquitin, along with 2.5 μg of a plasmid expressing GFP, GFP-TOPORS, or a GFP-TOPORS mutant (S98A or S98D). Cell lysates were analyzed by SDS-PAGE and immunoblotting with antibodies recognizing polyhistidine (top panel), TOPORS (middle panel), or actin (bottom panel). (B) HEK293 cells were transiently transfected with 2.5 μg of a plasmid expressing GFP, GFP-TOPORS, or a GFP-TOPORS mutant (S98A or S98D). Cell

lysates were analyzed by SDS-PAGE and immunoblotting with antibodies recognizing ubiquitin (top panel), TOPORS (middle panel), or actin (bottom panel). (C) Cell lysates were analyzed as described in (B). The amount of lysate in each lane was adjusted to equal amounts of TOPORS protein. (D) Cells transfected with TOPORS wild and phosphomimic mutant (S98D) were left untreated or treated with 2.5μM MG132 after 24 hours of the incubation for 4 hours. Cell lysates were prepared and subjected to immunoblotting using TOPORS and actin antibodies.

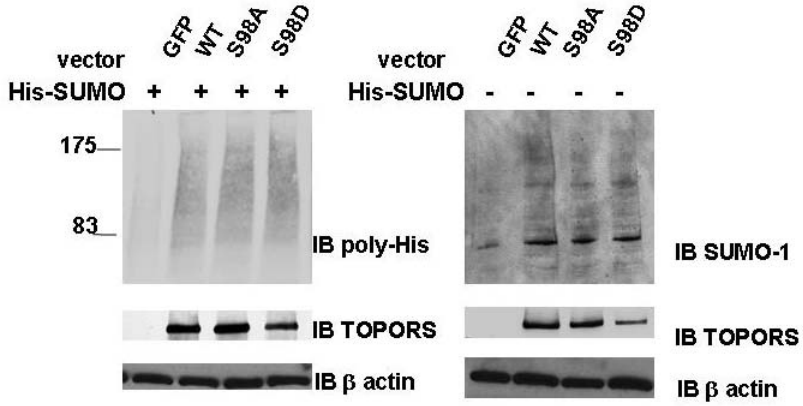

Figure 8. Effects of phospho-deficient (S98A) and phosphomimic (S98D) mutants on the SUMO E3 ligase activity of TOPORS in cells. In the left panel, HEK 293 cells were transiently transfected with 500 ng of a plasmid expressing polyhistidine-tagged SUMO-1, along with 2.5 μg of a plasmid expressing GFP, GFP-TOPORS, or a GFP-TOPORS mutant (S98A or S98D). Cell lysates were analyzed by SDS-PAGE and immunoblotting with antibodies recognizing polyhistidine (top panel), TOPORS (middle panel), or actin (bottom panel). In the right panel, HEK293 cells were solely transfected with 2.5 μg of a plasmid expressing GFP, GFP-TOPORS, or a GFP-TOPORS mutant (S98A or S98D). Cell lysates were analyzed in the same manner except for top panel in that antibodies recognizing SUMO-1 was used.

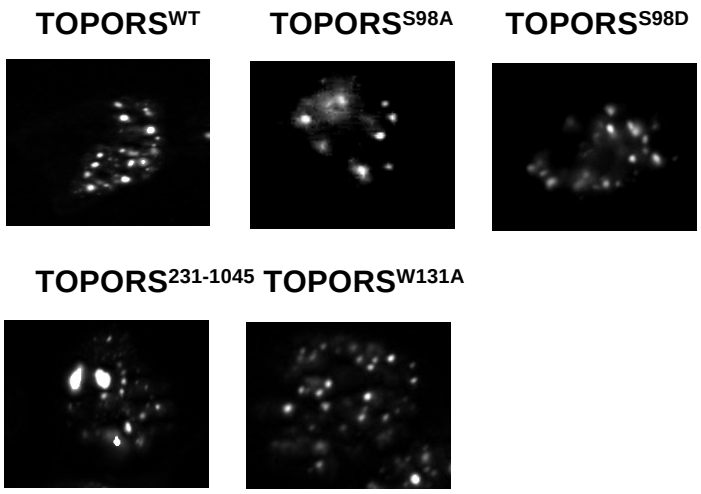

Figure 9. Mutation of Ser98 does not change the subcellular localization of TOPORS. HEK393 cells were transfected with plasmids encoding GFP-tagged TOPORS (wild type, S98A or S98D mutant, 231-1045 fragment, and W131A mutant) Transfectants were examined via fluorescent microscopy. Shown are representative transfected cells.

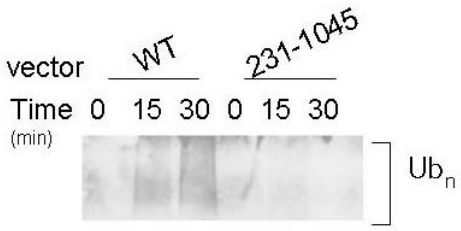

Figure 10. Time course of ubiquitin chain formation by TOPORS *in vitro* ubiquitin assay. Ubiquitination reactions were performed in a single tube, in the presence of 3nM of GST-TOPORS (wild and residues 231-1045) and presence of ATP regenerating system. Corresponding ubiquitin reactions were used as described in method section. The reactions were terminated at indicated time points, resolved in SDS-PAGE, analyzed by ubiquitin antibodies.

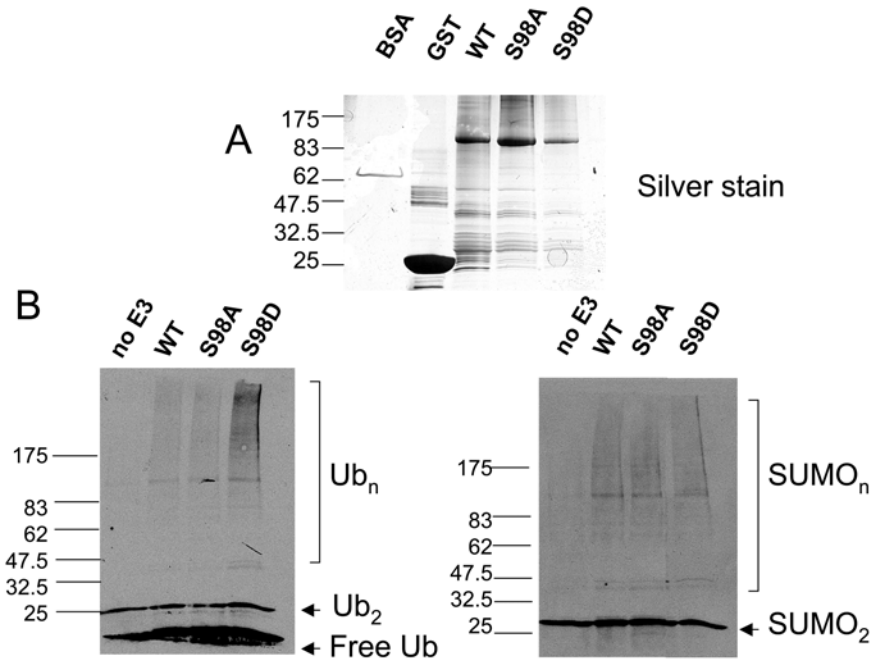

Figure 11. Effects of phospho-deficient (S98A) and phosphomimic (S98D) mutants on TOPORS ubiquitin and SUMO E3 ligase activity in vitro. (A) GST-TOPORS (wild, S98A or S98D mutants) from whole cell lysates from a bacterial strain was purified using GST pull down and eluted by buffer containing 50mM Glutathione. The eluted TOPORS proteins were visualized by silver stain. (B) Phosphorylated Ser98 is involved in TOPORS ubiquitin ligase activity. In vitro dual ubiquitin/ SUMO ligase reactions were performed using an ATP regenerating system with either no E3, wild, phospho-deficient (S98A) or phosphomimic mutant (S98D) GST-TOPORS. Concentrations of the E1, E2, modifier (ubiquitin or SUMO-1) were 150nM, 250nM, 25mM, respectively. Reaction products were divided into two equal portions, and ran on

SDS-PAGE and analyzed by immunoblotting with ubiquitin (B (a)) and SUMO-1 (B (b))antibodies

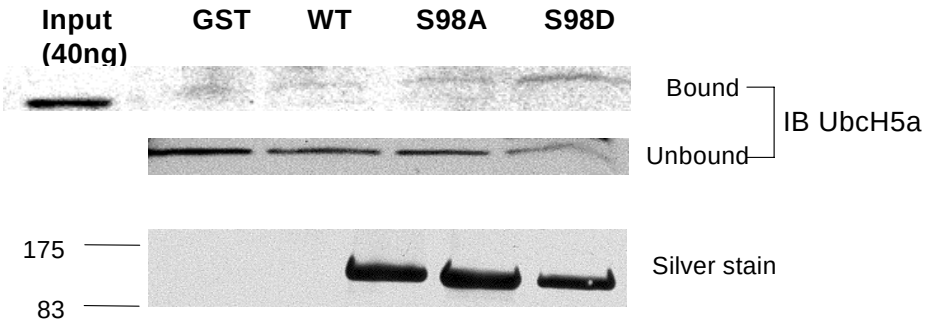

Figure 12. Mutation of Ser 98 to aspartate increases UbcH5a binding to TOPORS. Input (UbcH5a) represents 100% of input used in the binding assay. The binding assay was done at room temperature for 30 minutes.

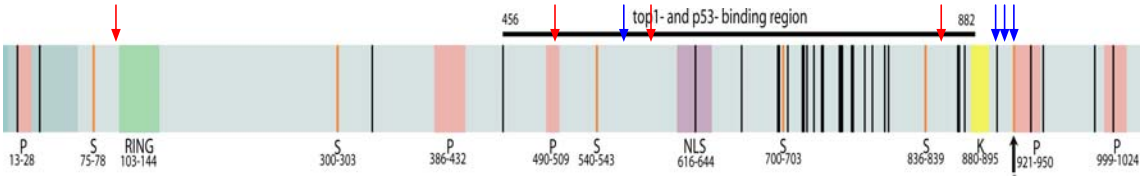

Figure 13. Identified post translational modification sites of TOPORS. Identified phosphorylated residues (i.g. S98, S499, S585 and S866) by our group are indicated by arrows in red, and reported identified post translational modification (e.g. SUMOylation at K560, phosphorylation at S904, S914 and S918) are indicated by blue arrows. P; PEST sequence (pink box), S; SUMO conjugation sites (magenta box), RING; RING domain (green box), NLS; nuclear localization signal (gray box), K; Lys-rich region (yellow box), black bars; SR/RS dipeptide repeats. The region identified as binding topoisomerase I and p53 using yeast two hybrid assays is also indicated.

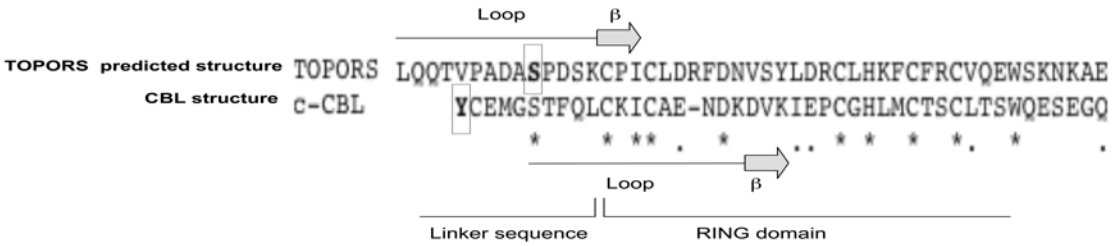

Figure 14. Similarity of the phosphorylated residues adjacent to the RING domains of TOPORS and CBL. Phosphorylated Tyr371 of c-Cbl and the putative phosphorylated Ser98 of TOPORS are shown in bold and are boxed. The structure of c-Cbl linker and the RING domain has been described previously (Zheng et al., 2000). Asterisks indicate identical amino acids. Dots indicate amino acids with similar characteristics. The putative structure of TOPORS in this region was determined by a prediction algorithm (www.predictprotein.org).

Figure 15. Alignment of TOPORS orthologs residues SP motif. SP motif is boxed. *H. sapiens, X. tropicalis, M.musculus, P. troglodytes, D. melanogaster* TOPORS orthologs were aligned using a ClustalW algorithm. Asterisks indicate identical amino acids.

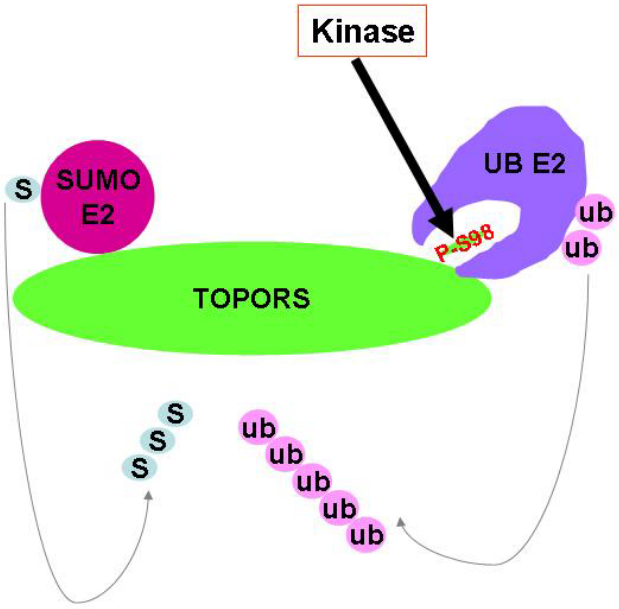

Figure 16. Model of TOPORS ubiquitin E3 ligase that is enhanced by phosphorylation of S98. TOPORS is shown in green, which interacts with UbcH5a, the ubiquitin- E2 enzyme, shown in purple. Phosphorylated Ser98 increases the E2 binding affinity. TOPORS SUMO E3 ligase activity is unaffected.

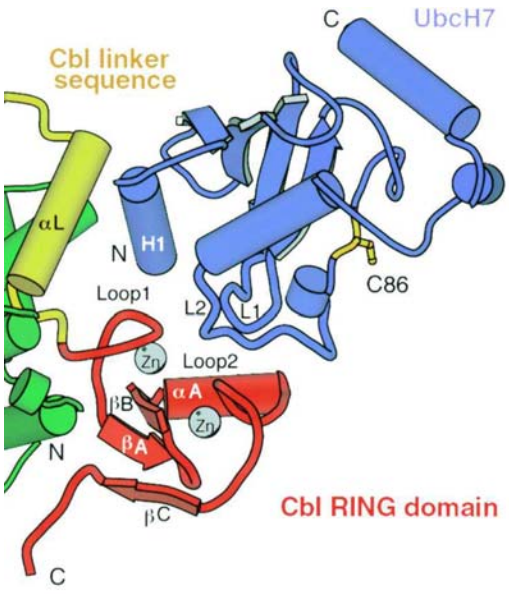

Figure 17. The ternary Complex of c-Cbl and UbcH7. Two UbcH7 loops pack in a shallow groove on the c-Cbl RING domain and a UbcH7 helix interacts with the c-Cbl linker helix (Zheng et al., 2000).

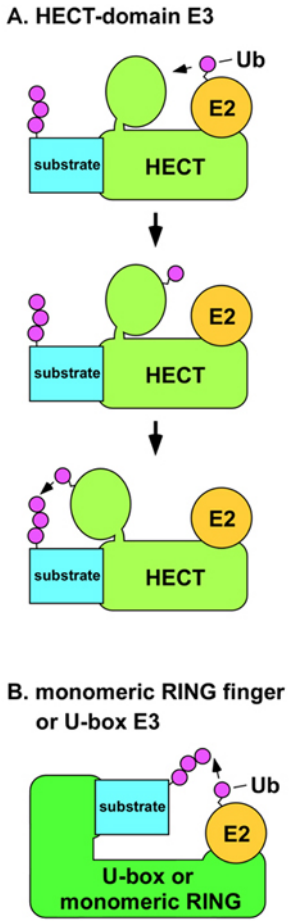

Figure 18. Model of HECT-domain and RING-domain E3 complex. (A) Model of HECT-domain E3 complex. The E2 binds N-terminal lobe of the HECT domain of E3 (top panel) and transfers Ub to C-terminal lobe via a thiolester bond (middle panel). C-terminal lobe swivels on a hinge-loop and catalyzes the transfer of Ub to the substrate (bottom panel). (B) Model of monomeric RING finger E3 (or U-box E3). The RING

finger (or U-box) E3 does not form a thiolester intermediate with ubiquitin but help to transfer ubiquitin from the E2 to the substrate (Kipreos, 2005)

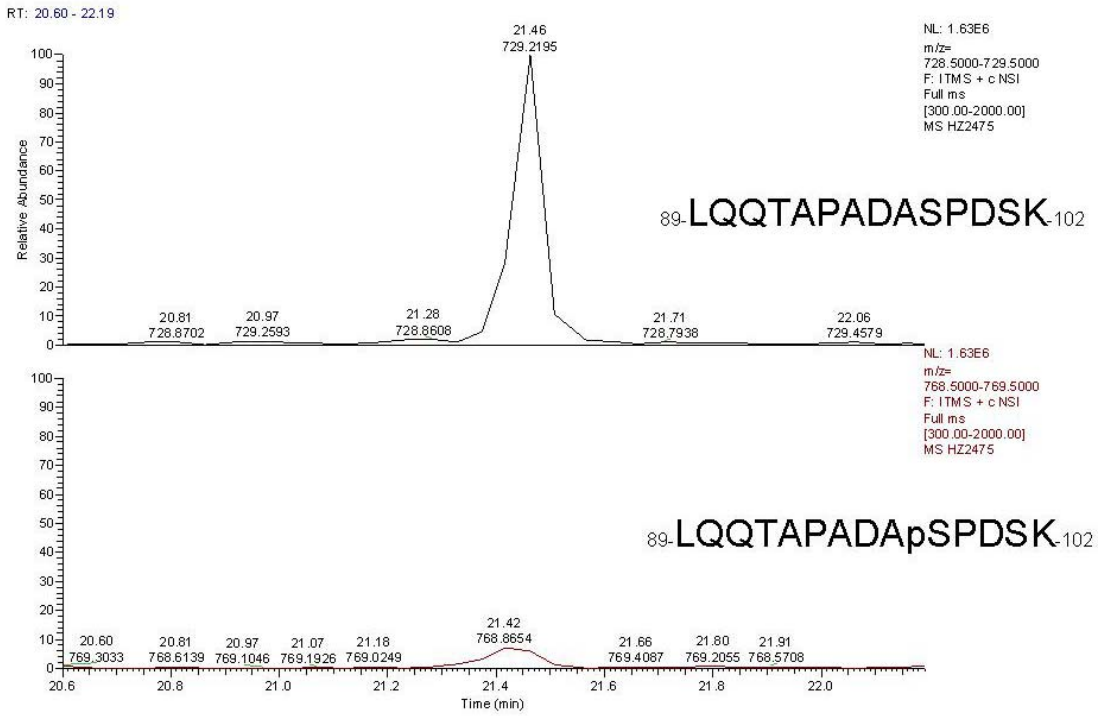

Figure 19. Peak intensity of unphosphorylated and phosphorylated ^{89}LQQTAPADASPDSK^{102}. A phosphorylation level of <10% of the corresponding unphosphorylated peptides (residues 89-102) was estimated based on relative peak intensities as observed by LC-MS/MS analyses of TOPORS peptides

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
DMEM	Dulbecco's Modified Eagle Media
DTT	Dithiothreitol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
EDTA	Ethylendiaminetetraacetic acid
FBS	Fetal bovine serum
GFP	Green Fluorescence Protein
GST	Glutathione-S-transferase
IgG	Immunoglobulin G
Kb	Kilobase (1000 bases)
kDa	Kilodalons (1000 daltons)
NB	nuclear blody
NP-40	Nonidet P40 (IGEPAL CA-630)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEST	proline-, glutamate-, serine- or threonine-rich sequence
PML	Promyelocytic leukemia protein
PMSF	Phenylmethyl-sulfonyl fluoride
RING	Really interesting new gene

RS	arginine-serine or serine-arginine dipeptide repeats
SAE2	SUMO activating enzyme
SUMO-1	Small ubiquitin like modifier
Tris-HCl	Tris [hydroxymethyl] aminomethane hydrochloride
Top1	Topoisomerase I (human)
TOPORS	Topoisomerase I-binding, arginine- and serine-rich protein
Ub	Ubiquitin
Ubc	Ubiquitin conjugating enzyme

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