Analysis of functional domains required for hRad18 interactions with HHR6B and hUbc9

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Saskatoon

By

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ABSTRACT

DNA post-replication repair (PRR) is a cellular tolerance mechanism by which eukaryotic cells survive lethal lesions during or after DNA synthesis. In the yeast *Saccharomyces cerevisiae*, modification of proliferating cell nuclear antigen (PCNA) by ubiquitin and by small ubiquitin-like modifier (SUMO) plays an important role in PRR. PCNA ubiquitination is dependent on Rad6, a ubiquitin-conjugating enzyme (E2) and Rad18, a ubiquitin ligase (E3). Rad6 and Rad18 form a stable complex. PCNA sumoylation is dependent on Ubc9, an E2 specific to SUMO modification.

PRR in mammalian cells is less well understood. However, human Rad18 (hRad18) has been found to interact with human Rad6 (HHR6A/B). In this study, we detected physical interaction between hRad18 and human Ubc9 (hUbc9) through yeast two-hybrid assays. In order to define the domain(s) of hRad18 involved in the formation of a complex with HHR6B or hUbc9, a series of yeast two-hybrid constructs containing various *hRAD18* gene deletions and mutations were made. A C-terminal region of hRad18, containing the putative HHR6A/B binding domain (amino acids 340 to 395), interacts with HHR6A/B while the N-terminus (amino acids 1-93) does not. Yeast Rad18 has a homologous fragment of the HHR6A/B binding domain and this fragment is sufficient to interact with yeast Rad6 in yeast two-hybrid assays, so we infer that hRad18 interacts with HHR6B through the same domain. Surprisingly, both the N-terminal and C-terminal fragments of hRad18 can interact with hUbc9, suggesting the existence of two

separate domains in hRad18 interacting with hUbc9. The N-terminal fragment of hRad18 contains only a RING finger domain (amino acids 25-64), which is probably responsible for binding to hUbc9. The C-terminal fragment of hRad18 with HHR6A/B binding domain deletion can still interact with hUbc9, suggesting that the HHR6A/B binding domain is not involved in hUbc9 interaction. A key cysteine mutation (C28F) in the RING finger domain abolished the interactions of hRad18 with both HHR6A/B and hUbc9. This amino acid substitution is likely to alter the three-dimensional structure of the protein, thus making the protein unstable. Taken together, results obtained from this study suggest that hRad18 may regulate the modification status of PCNA by interacting with two different E2s, HHR6A/B and hUbc9, through distinct domains.

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List of Abbreviations

3-AT	3-aminotriazole
aa	amino acid
AP site	apurinic or apyrimidinic site
APG12	Autophage-12
APS	Ammonium persulfate
BER	base excision repair
β-gal	β-galactosidase
bp	base pair
CIP	calf intestinal alkaline phosphatase
Cys	cysteine
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DSBs	double stranded breaks
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
GST	Glutathione S-transferase
His	histidine
HR	homologous recombination
Hub1	Homologous to Ub
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	ionizing radiation
ISG15	Interferon stimulated gene of 15 kDa
Kb	kilobase pair
kDa	kiloDalton

Leu	leucine
Lys	lysine
MCS	Multiple cloning sites
Met	methionine
MMS	methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mRNA	messenger RNA
NER	nucleotide excision repair
OD	optical density
ORF	open reading frame
PCNA	Proliferating cell nuclear antigen
PEG	polyethylene glycol
Phe	Phenylalanine
PRR	postreplication repair
rpm	revolutions per minute
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SD medium	synthetic dextrose minimal medium
SDS	sodium dodecyl sulfate
Ser	serine
SSBs	single strand breaks
ssDNA	single stranded DNA
SUMO	small ubiquitin-like modifier
TEMED	N,N,N',N'-Tetramethylethylenediamine
Trp	tryptophan
Ub	ubiquitin
Ubl	Ub-like modifier proteins

Ura	uracil
UV	ultraviolet
WT	wild type
XP	Xeroderma Pigmentosum
YPD medium	yeast extract/peptone/dextrose medium

CHAPTER ONE

INTRODUCTION

1.1 Ubiquitination and sumoylation

1.1.1 Ubiquitin and Ub-like modifier proteins

Ubiquitin (Ub), a small protein that can be covalently linked to itself or other proteins, was discovered in the mid-1970s (Schlesinger *et al.*, 1975). The first function attributed to Ub was the proteasome-dependent degradation of short-lived proteins in mammalian cells (Ciechanover *et al.*, 1984; Finley *et al.*, 1984). Since then, the research in this area exploded and Ub has been associated with nearly every aspect of eukaryotic cell biology (Aguilar and Wendland, 2003; Glickman and Ciechanover, 2002; Welchman *et al.*, 2005). The breadth of biological roles for ubiquitination made it one of the busiest activities in cells.

Ub is one member of a family of proteins that have remarkably similar structures, but variable sequences. These Ub-like modifier proteins (Ubl) share common characteristics with Ub. Most of these proteins are expressed as fusion products which are subsequently processed to become the mature form by isopeptidase cleavage, exposing a signature diglycine sequence at the C-terminus (Amerik and Hochstrasser, 2004; Melchior *et al.*, 2003). When conjugated to a substrate, the terminal glycine is used to form an isopeptide bond with an amino group, usually a lysine residue on the target protein. Some proteins in this family are listed in **Table 1-1**.

Table 1-1 Ubl proteins and their functions

Modifier protein	Function	References
SUMO (SMT3 in yeast)	Targets proteins to the nucleus frequently involved in regulating transcription	(Kim *et al.*, 2002)
Nedd8 (Rub1 [related to Ub] in yeast)	Regulates the SCF (Skip1/Cullin/F-box) Ub ligases	(Schwechheimer and Deng, 2001)
Hub1 (homologous to Ub)	Plays a role in cell polarity processes in yeast	(Dittmar *et al.*, 2002)
ISG15 (interferon stimulated gene of 15 kDa)	Implicated in the regulation of interferon signaling	(Malakhova *et al.*, 2002)
APG12 (autophage-12)	Regulates the ‘cytoplasm-to-vacuole’ targeting and autophagy pathways	(Khalfan and Klionsky, 2002)

From Aguilar and Wendland (2003)

Among all members of Ub family, Ub is studied most and its function is well understood. This small 76 amino acid protein is found throughout eukaryotic cells and is highly conserved, with only three amino-acid differences between yeast and humans. This remarkable conservation reflects the importance of Ub's biological functions in eukaryotic cells.

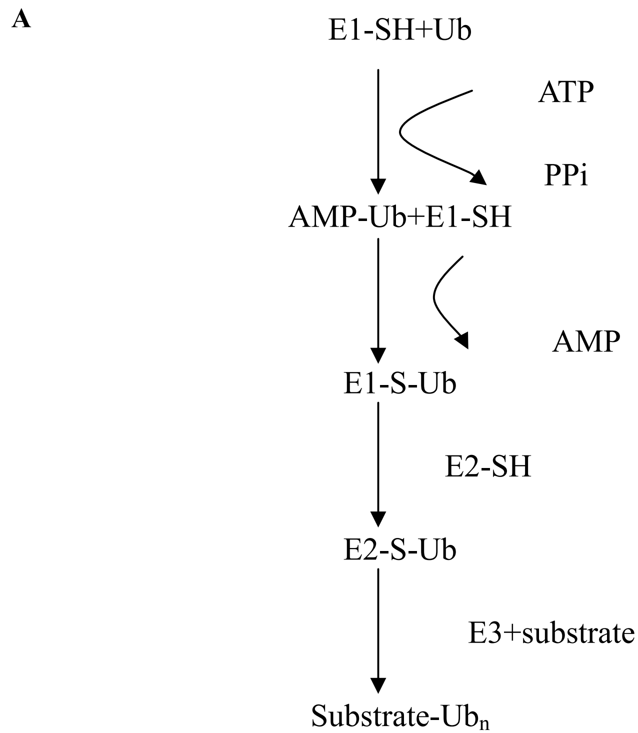
1.1.2 Ubiquitination components

The conjugation of Ub to substrates is completed through three steps (Fig. 1-1 A and B) involving three different enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and Ubiquitin-ligase (E3). First, a thioester bond between the C-terminal glycine of Ub and the active cysteine of E1 is formed in an ATP-dependent manner. Ub is then transferred to E2, again through a thioester linkage. Finally, an E3 catalyzes the formation of an isopeptide bond between the C-terminus of Ub and a lysine residue of a specific target protein. Additional Ub moieties can be conjugated to Lys48 (Chau *et al.*, 1989; Hershko and Heller, 1985) or Lys63 (Arnason and Ellison, 1994; Spence *et al.*, 1995) on previous Ub to form a polyubiquitin chain.

1.1.2.1 E1s

During ubiquitination, the first task is to activate the C-terminus of Ub, thus making it capable of conjugating to a substrate. This reaction is catalyzed by an E1 enzyme in two steps. Firstly, a Ub-adenylate intermediate is formed in which the

A



B

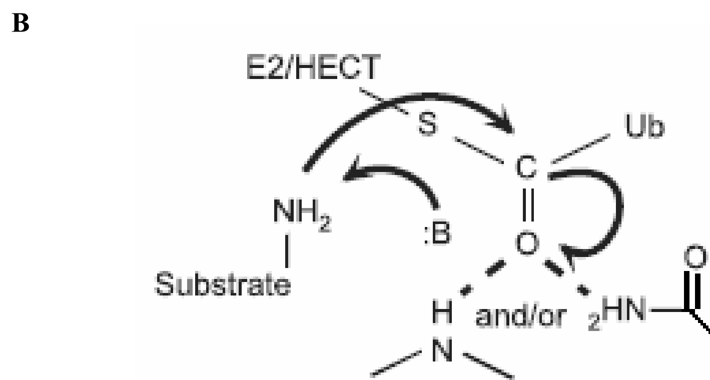


Fig. 1-1 Basic steps in ubiquitination. (A) The conjugation of Ub to a substrate usually involves three steps: an initial activation step catalyzed by E1, an intermediate step in which Ub is covalently linked to an E2, and a final step in which Ub reaches the substrate. (B) Anticipated catalysis of Ub conjugation to substrate. B: denotes a general base which is needed to deprotonate the lysine residue on substrate. Adapted from Pickart (2004).

C-terminal carboxyl group of Ub is covalently linked to AMP. Breakage of the Ub-AMP linkage is followed by the formation of an E1-Ub thiol ester with the C-terminus of Ub linking to a cysteine residue on E1. There is only one E1 in yeast for ubiquitination (Ciechanover *et al.*, 1982), which suggests the first step in ubiquitination is performed in a same way though there are lots of ubiquitination substrates. In *Saccharomyces cerevisiae*, the E1 is Uba1, a 114 kDa protein (McGrath *et al.*, 1991). *UBA*1 gene is an essential gene. In general, each Ubl has a single dedicated E1.

1.1.2.2 E2s

The next step in the ubiquitination process is the transfer of Ub from the E1 cysteine residue to an E2 cysteine. There is a large family of E2s dedicated to ubiquitin, comprising 11 enzymes in *S. cerevisiae* (**Table 1-2**) and many more in higher organisms (Pickart, 2001). Each E2 may serve several E3s. The number of E3s is much larger. Each E3 cooperates with one or a few E2s (Chen *et al.*, 1993; Sommer and Wolf, 1997) to recognize specific substrate(s). Different combinations of E2 and E3 lead to the large number and extraordinary diversity of ubiquitination substrates.

All E2s, whether dedicated to Ub or other Ubls, share a conserved core domain of approximately 150 amino acids. The core domain consists of four standard helices, a short 3_{10} helix, and a four-stranded antiparallel β-sheet (VanDemark and Hill, 2002). The β-sheet and α2 form a central region that is bordered by α1 at one end and α3-α4 at the other (Fig. 1-2). The E2 active site cysteine, which is absolutely conserved, sits in a

shallow cleft on the protein surface. Not surprisingly, many of the most highly conserved E2 residues surround the active cysteine (Cook *et al.*, 1994; Jiang and Basavappa, 1999; Worthylake *et al.*, 1998). Some of these residues interact with Ub, and others presumably interact with E1. Most of the poorly conserved E2 residues cluster on the opposite side of the active cysteine. Some E2s also have N-terminal or C-terminal extensions, which are believed to mediate interactions between E2s and downstream factors: E3s or substrates (Pickart, 2001). The extensions make these E2s distinct, thus achieving the specificity and diversity of E2-E3 interaction in spite of the core structure similarity of all E2s.

The instability of E2-Ub thiol ester hinders the investigation of physical interaction between these two proteins. However, an NMR (nuclear magnetic resonance) analysis of chemical shift perturbations during yeast Ubc1-Ub thiol ester formation successfully defined the E2-Ub interface (Hamilton *et al.*, 2001). The result showed that the C-terminus of Ub adopts a partially extended conformation that wraps around part of the E2 surface which is proximal to the active cysteine site. This interface does not overlap the site where E3 may bind. The conservation of the E2 core domain suggests a similar manner by which other E2s interact with Ub.

The crystal structures of E2-E3 complexes helped us understand how E3s select their E2s (Huang *et al.*, 1999; Zheng *et al.*, 2000). The structures suggest that most E2s contact their cognate E3s through side chains at the C-terminal end of E2 α1, the loop between β1 and β2, and the distal end of the active site loop. Some E2-E3 complexes may involve E2 terminal extensions (Madura *et al.*, 1993; Xie and Varshavsky, 1999).

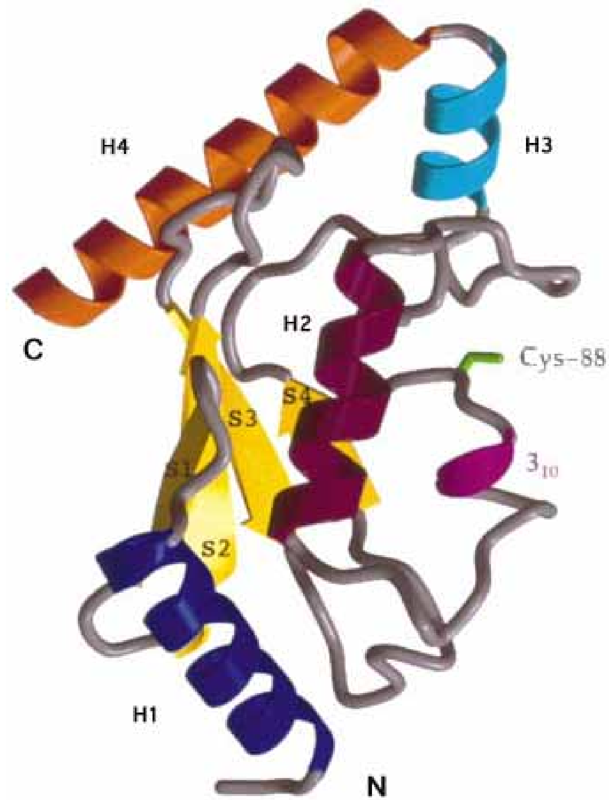


Fig. 1-2 Yeast Ubc2 (Rad6) structure. All E2s share a conserved core domain which consists of four standard helices, a short 3_{10} helix, and a four-stranded antiparallel β–sheet. The active site cysteine (C88) is shown in green. Adapted from Worthylake (1998).

TABLE 1-2 *S. cerevisiae* E2 enzymes

E2	Cognate E3	Function(s)
Ub E2s		
Ubc1	Unknown	Sporulation: essential in *ubc4Δ ubc5Δ* cells (Hochstrasser, 1996)
	Unknown	Endoplasmic reticulum (ER) degradation (Friedlander *et al.*, 2000)
Ubc2/Rad6	Ubr1	N-end rule proteolysis (Dohmen *et al.*, 1991)
	Bre1	H2B ubiquitination (Joazeiro and Weissman, 2000)
	Rad18	PRR (Bailly *et al.*, 1994)
Ubc3/Cdc34	SCF^{Cdc4}	Regulates G1/S transition (Deshaies, 1999)
	SCF^{Grr1}	Cell cycle (Deshaies, 1999)
	SCF^{Met30}	Transcription regulation (Kaiser *et al.*, 2000)
Ubc4, Ubc5	Ufd4	protein degradation (Johnson *et al.*, 1995)
	Rsp5	DNA damage response (Huibregtse *et al.*, 1997)
	Unknown	Mitotic transition (Seino *et al.*, 2003)
Ubc6	Unknown	Integral protein of ER membrane (Sommer and Jentsch, 1993)
	Doa10	ER degradation (Swanson *et al.*, 2001)

Ubc7	Der3	ER degradation (Bordallo *et al.*, 1998)
	Doa10	ER degradation (Swanson *et al.*, 2001)
	Teb4	ER degradation (Hassink *et al.*, 2005)
Ubc8	RLIM	Histone deacetylase2 degradation (Kramer *et al.*, 2003)
		Glucose-induced proteolysis (Schule *et al.*, 2000)
		ISGylation (Kim *et al.*, 2004)
Ubc10	Unknown	Peroxisome biogenesis (Wiebel and Kunau, 1992)
	Unknown	Cell cycle (Liu *et al.*, 2004)
Ubc11	Unknown	Mitotic transition (Seino *et al.*, 2003)
Ubc13	Rad5	PRR (Hofmann and Pickart, 1999; Ulrich and Jentsch, 2000)
Other E2s		
Ubc9	Siz1/2	Sumoylation (Johnson and Blobel, 1997; Saitoh *et al.*, 1998)
Ubc12	Unknown	Nedd8 modification (Gong and Yeh, 1999; Wada *et al.*, 2000)

Less is known about the interface of E1-E2 interaction. E1 may partially share the E3 binding sites of E2 (Pickart and Eddins, 2004). Studies of E2 interaction with Ub, E1 and E3s can help us understand the biological significance of E2 existence as an intermediate and how Ub is transferred from E1 to E2, then E2 to E3, and finally elucidate the entire process of ubiquitination.

1.1.2.3 E3s

The last step in ubiquitination is the covalent ligation of one or more Ub molecules to the substrate mediated by an E3. There are many E3s in higher organisms, ranging from several hundred to over a thousand (Pickart and Eddins, 2004). The large number of E3 reflects the breadth of ubiquitination involved in biological functions. Different from E2s, E3s are structurally diverse. Nevertheless, to date, all known E3s belong to only three protein families: **H**omologous to **E**6AP **C**arboxy **T**erminus (HECT), **R**eally **I**nteresting **N**ew **G**ene (RING), and Ufd2 (**U**b **f**usion **d**egradation protein 2) (Johnson *et al.*, 1995) homology (U-box) proteins.

1.1.2.3.1 HECT domain E3s

This domain was first revealed in **E**6 **A**ssociated **P**rotein (E6AP). E6AP forms a complex with E6 protein of oncogenic **h**uman **p**apilloma**v**irus (HPV). This complex functions as an E3 that binds and ubiquitinates the host protein p53, resulting p53 degradation, viral DNA replication, and attendant deleterious consequences for the host

cell (Huibregtse *et al.*, 1994). The final third of the E6AP is 35-45% identical to numerous other proteins (Huibregtse *et al.*, 1995). HECT E3s are defined by the presence of this ~350 amino acid C-terminal region. Within this region, there is a strictly conserved cysteine residue positioned ~35 residues upstream of the C-terminus. HECT E3s employ a different mechanism from other type of E3s to facilitate Ub conjugation by forming a thiol ester intermediate with Ub through this conserved cysteine. All HECT E3s function in a similar way in ubiquitination: the N-terminus of HECT E3s mediates substrate recognition, while the HECT domain binds the E2-Ub intermediate and further catalyzes Ub covalent ligation to substrate (Pickart *et al.*, 2001).

Mutations in the *E6AP* gene cause Angelman syndrome, an inherited disease characterized by severe mental retardation (Kishino *et al.*, 1997; Matsuura *et al.*, 1997). So E6AP must have some other substrates in HPV uninfected cells. Some substrates were identified, including Src family tyrosine kinases Blk and Src, nucleotide excision repair factor HHR23A, and Mcm7, a subunit of replication licensing factor (Pickart, 2001). But it is unknown whether these substrates are related to Angelman syndrome.

The crystal structure of the E6AP-HECT-UbcH7 complex (Huang *et al.*, 1999) showed that the HECT domain is L-shaped, with the active cysteine positioned at the junction of the two arms. Its final 100 residues form the short arm while the N-terminal part of the HECT domain forms the long arm (Fig. 1-3). The UbcH7 binds at one end of the long arm. In this structure, the cysteines of the E2 and E3 are separated far away. People assume that catalytic transfer of Ub from E2 to E3 involves large-scale

conformational transitions. The assumption is supported by the structure of the HECT domain of WWP1/AIP5, which is folded into an inverted T shape (Fig. 1-4) in which the cysteines of the E2 and E3 are much closer (Verdecia *et al.*, 2003). Further conformational transitions would allow the Ub transfer to occur.

Although the structure of the WWP1/AIP5 HECT domain is known, this protein's biological functions remain obscure and its *in vivo* substrates are yet to be determined. This is true for the majority of HECT E3s. Aside from E6AP, the best-characterized HECT E3 is the essential yeast enzyme Rsp5. This E3 contains a C2 lipid binding domain for localization to the plasma membrane and three WW domains which bind protein's proline-rich regions (Chen and Sudol, 1995). Whether it contains WW domains subdivides HECT E3s into those possessing WW domains, and those lacking WW domains, for example E6AP. Rsp5 has a wide range of substrates (Pickart, 2001), reflecting HECT E3s' diverse biological functions.

1.1.2.3.2 U-box E3s

The U-box (**U**fd2-homology domain) was first identified in yeast Ufd2, a protein involved in UFD (**U**b **f**usion **d**egradation) pathway (Johnson *et al.*, 1995). Ufd2 was classified as an E4 because it lacks its own substrate and instead catalyzes the polyubiquitination of another E3's substrate (Koegl *et al.*, 1999). Later studies showed that some other U-box proteins can mediate ubiquitination in the presence of E1 and E2

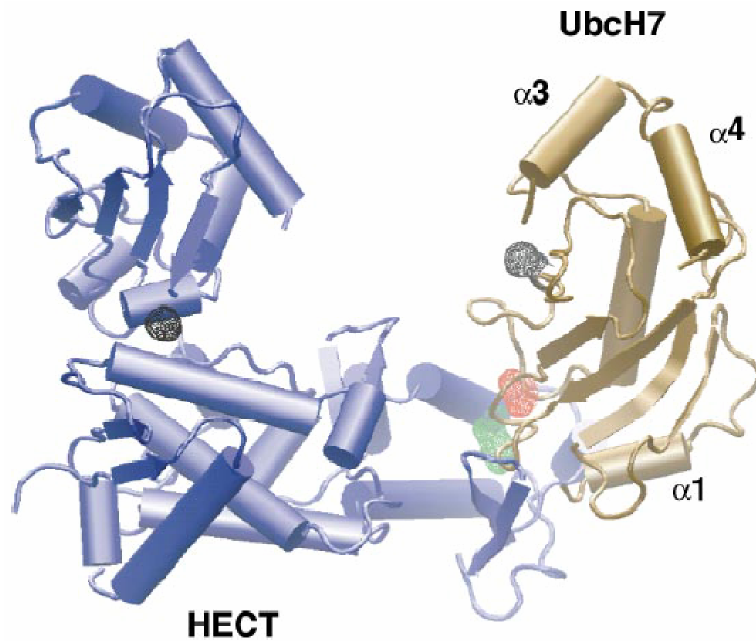


Fig. 1-3 HECT-UbcH7 structure. The HECT domain of E6-AP is shown in blue, with side chain of C820 (black) highlighted. UbcH7 is shown in ochre, with the side chains of F63 (green), C86 (black), and P97 (red) highlighted. Adapted from Huang (1999) and Pickart (2001).

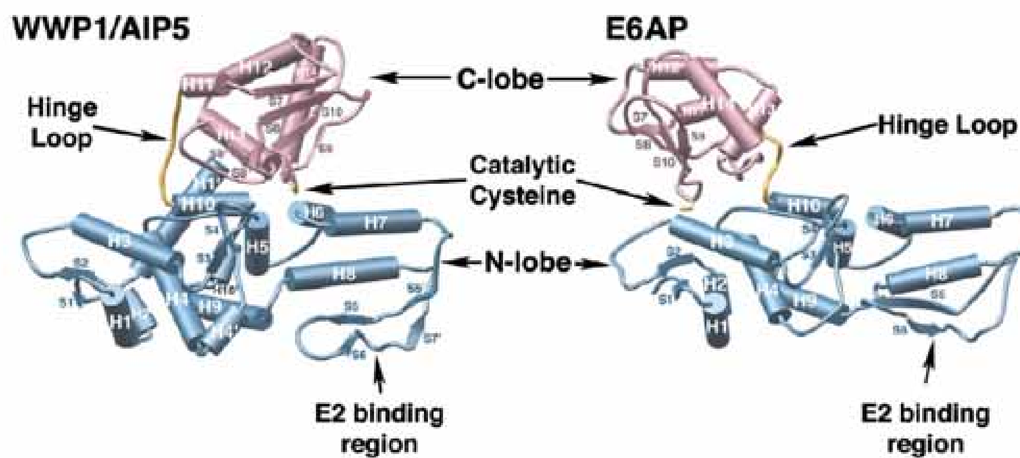


Fig. 1-4 Comparison of the HECT Domains of WWP1/AIP5 and E6AP. The structure of the HECT domain of WWP1/AIP5 consists of two lobes connected by a hinge loop (residues 803–806) colored gold. The N lobe consists of residues 546–802 and is colored blue. The C lobe, comprising residues 807–917, is colored rose. Adapted from Verdecia (2003).

and in the absence of E3 (Hatakeyama *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001).

The U-box is a domain of *ca.* 70 amino acids. The structure of the U-box in the essential *S. cerevisiae* pre-mRNA splicing factor Prp19 is remarkably similar to the structure of the RING domain (Fig. 1-5) (Ohi *et al.*, 2003). The conserved zinc-binding sites supporting the cross-brace arrangement in the RING finger are replaced by hydrogen-bonding networks in the U-box. Just as the mutation of a zinc-coordinating residue leads to RING domain unfolding, the mutation of key U-box residues involved in hydrogen bonding cause U-box unfolding (Ohi *et al.*, 2003). Deletion of the U-box or mutation of conserved amino acids within it abolishes ubiquitination activity (Hatakeyama *et al.*, 2001). Mutations of amino acids which destabilize the tertiary structure of U-box also eliminates its E3 activity (Ohi *et al.*, 2003). These observations suggest that U-box proteins are indeed E3s. Some of U-box proteins function as E4s to mediate the assembly of polyubiquitin chains on proteins ubiquitinated by another E3 enzyme (Imai *et al.*, 2002; Koegl *et al.*, 1999). This suggests that E4 activity may be a common feature of U-box proteins.

1.1.2.3.3 RING finger E3s

It is not clear whether all RING finger proteins play roles in ubiquitination. However, a large number of these proteins are E3s and they comprise the largest known class of E3s. The RING finger domain can be defined by exist of the consensus sequence

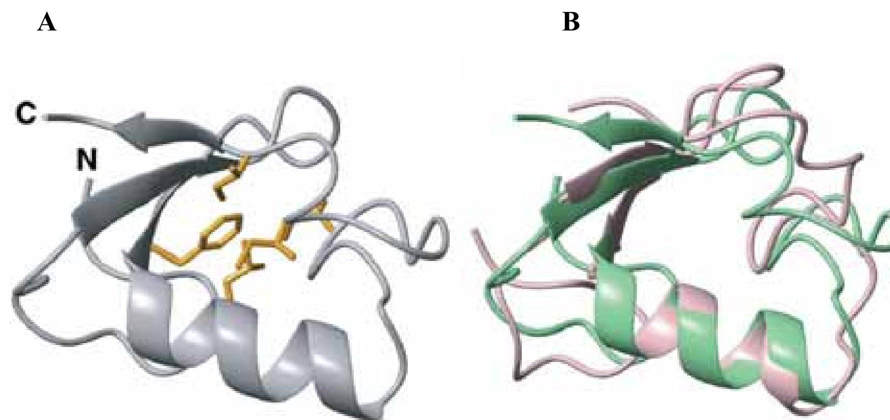


Fig. 1-5 The U-box and RING finger domain share a conserved fold. A, the structure of the Prp19 U-box domain, with core hydrophobic residues in yellow. B, Overlay of the structures of the Prp19 U-box (green) and the c-Cbl RING finger (pink). Adapted from Ohi (2003).

Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}- Cys/His-X_2-Cys-X_{4-48}-Cys-X_2-Cys, where X is any amino acid, Cys and His represents zinc binding residues (Joazeiro and Weissman, 2000). Every RING motif coordinates two zinc atoms with each atom ligated tetrahedrally by either four cysteine, or three cysteines and a histine, in a unique cross-brace arrangement. One feature of RING domains is that they can directly bind E2s. The structure of one E2-E3 complex revealed that no RING domain side chain comes closer than ~15 Å to the E2 active site cysteine (Zheng *et al.*, 2000). This observation and other structures (Orlicky *et al.*, 2003; Zheng *et al.*, 2002) suggest that RING E3s function in ubiquitination as molecular scaffolds that bring together the substrate lysine and the E2-Ub intermediate.

RING E3s can be classified into two types: single-subunit and multi-subunit. Single-subuint RING E3 is a polypeptide containing a RING finger domain (no two or more RING domains protein reported) and other motifs necessary for E3 activity. Multi-subunit RING E3s include **C**ullin-**R**ING **L**igase (CRL) and **A**naphase-**p**romoting **C**omplex/**C**yclosome (APC/C). CRLs are composed of several proteins in which cullin and RING domain subunit Rbx1 (also known as Roc1 or Hrt1) (Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Tan *et al.*, 1999) are core members. Human cells express seven different cullins (Cul 1, 2, 3, 4A, 4B, 5 and 7) (Petroski and Deshaies, 2005); each of them functions as a scaffold protein to recruit Rbx1 and adapter protein(s) to form a RING E3 complex. The adapter protein binds to the N-terminal region of cullins, whereas Rbx1 which recruits E2 binds to the C-terminal globular domain (Zheng

et al., 2002). This E3 complex recruits the substrate through an adapter protein or a substrate receptor which binds to an adapter protein. For example, Cul1 binds to Skp1 which in turn binds F-box substrate receptor (Schulman *et al.*, 2000). F-box protein contains an F-box motif, which is required for binding to Skp1, as well as a specific substrate-recognition motif. F-box proteins are highly variable and interchangeable. Thus, Cul1 can assemble with numerous substrate receptors to form CRLs that share a common catalytic core yet recruit different substrates (Fig. 1-6) (Nakayama *et al.*, 2001). Other cullins have a similar mechanism to assemble multi-subunit RING E3s (Petroski and Deshaies, 2005).

APC/C is a high molecular mass complex composed of at least 11 subunits, but it is only fully active as an E3 once it has bound to Cdc20, Cdh1, or related activators (Peters, 2002). Two of its subunits, APC2 and APC11, are distant members of the cullin and RING domain families, respectively (Yu *et al.*, 1998; Zachariae *et al.*, 1998).

1.1.3 Sumoylation

1.1.3.1 SUMO

Small **u**biquitin-like **mo**difier (SUMO) is a member of Ubl. SUMO is conjugated to a variety of cellular proteins. Sumoylation is most well characterized among all Ubl modifications.

In lower eukaryotes, such as yeast, insects, and nematodes, only one *SUMO* gene is expressed, whereas in mammalian cells, there are three paralogs SUMO-1, SUMO-2,

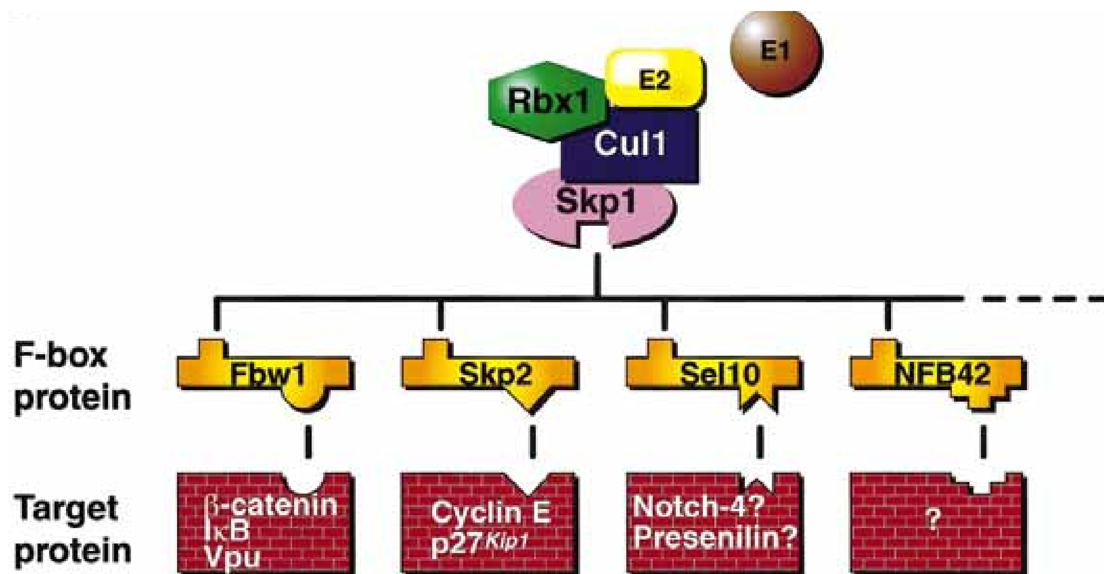


Fig. 1-6 SCF complex as a versatile Ub E3. Skp, Cul1, and Rbx1 are invariable components of the SCF complex. F-box proteins are highly variable and interchangeable. Mammals likely express at least several hundred F-box proteins including Fbw1, Skp2, Sel10, and NFB42. By changing F-box proteins, the SCF complex is thus able to change its substrate specificity. Adapted from Nakayama (2001).

and SUMO-3 (Lapenta *et al.*, 1997). SUMO-2 and SUMO-3 share 95% sequence identity, but are relatively different from SUMO-1 (50% sequence identity) (Lapenta *et al.*, 1997). Human SUMO-1 is 18% identical to Ub. SUMO-1 and Ub share the same fold organization and their tertiary structures are superimposable (Bayer *et al.*, 1998). SUMO-1 has a flexible, 21-amino acid N-terminal extension that is absent in Ub. The extension is rich in charged residues and may contribute to protein-protein interactions. In addition, the overall surface charge distribution is different for these two proteins (Bayer *et al.*, 1998). These differences likely reflect their divergent biological properties.

1.1.3.2 Sumoylation components

SUMO is covalently conjugated to substrates via a three-step enzymatic pathway analogous to that of ubiquitination. The E1 for SUMO is a heterodimer of SAE1 and SAE2 (known as Aos1 and Uba2 in yeast), two subunits with sequence similarity to the N and C termini, respectively, of Ub E1 (Desterro *et al.*, 1999; Gong *et al.*, 1999; Johnson *et al.*, 1997; Okuma *et al.*, 1999). The E2 for SUMO is Ubc9 (Desterro *et al.*, 1997; Johnson and Blobel, 1997). One feature of Ubc9 different from Ub E2s is that it can recognize substrate proteins directly. *In vitro* experiments showed that E1 and E2 are able to conjugate SUMO to substrates in the absence of E3 (Desterro *et al.*, 1999; Okuma *et al.*, 1999). Ubc9 usually interacts directly with SUMO substrates. The binding site has a consensus sequence of "ΨKxE", where Ψ is a large hydrophobic residue, K is the lysine to which SUMO is conjugated, x is any aa, and E is glutamic acid (Sampson *et al.*, 2001).

SUMO is modified to the lysine residue in this motif (Rodriguez *et al.*, 2001; Sampson *et al.*, 2001). Sometimes, Ubc9 binding (Buschmann *et al.*, 2001) and SUMO modification (Hay, 2005) happen on the site which does not conform to the consensus. SUMO-2/-3 each possess exposed SUMO modification consensus sequences that can be utilized to form polymeric SUMO chains (Tatham *et al.*, 2001). The precise function of poly-SUMO chains is unknown (Bylebyl *et al.*, 2003). Though only SAE1/SAE2 and Ubc9 can catalyze sumoylation, proteins that increase efficiency of SUMO conjugation were discovered, suggesting the existence of SUMO E3s. In *S. cerevisiae*, deletion of *SIZ1* and *SIZ2* genes eliminates almost all SUMO modification, indicating that these proteins function as SUMO E3 *in vivo* (Johnson and Gupta, 2001; Takahashi *et al.*, 2001). In higher eukaryotes the **p**rotein **i**nhibitor of **a**ctivated **S**TAT (signal transducer and activator of transcription) (PIAS) proteins are homologs of the Siz proteins and also appear to act as SUMO E3. Siz1, Siz2, and PIAS proteins have an unusual RING-related domain, termed the Siz/PIAS RING finger (SP-RING) (Hochstrasser, 2001). The SP-RING finger has the capacity to interact with Ubc9 and the substrate and thus can increase the rate of substrate sumoylation.

1.1.3.3 SUMO processing and deconjugation

SUMO-1 is translated as a 101-amino acid nonfunctional precursor which must be processed to remove the C-terminal four amino acids and expose the characteristic double glycine motif that is required for sumoylation (Johnson *et al.*, 1997; Kamitani *et al.*,

1997). This processing is carried out by SUMO-specific proteases that also remove SUMO from modified substrates. Two SUMO-specific proteases, Ulp1 and Ulp2, have been characterized (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000). Database searching identified eight human proteins with significant sequence homology to yeast Ulp1 (Yeh *et al.*, 2000), but not all of them are specific for SUMO (Hay, 2005).

1.1.3.4 Crosstalk between ubiquitination and sumoylation

In unstimulated cells, the transcription factor NF-κB is held in the cytoplasm in an inactive state by IκB inhibitor proteins. Activation of NF-κB is mediated by signal-induced degradation of IκBα, which allows the active transcription factor to translocate into the nucleus. Binding of NF-κB to its DNA recognition sites activates transcription from responsive genes. IκBα is also modified by SUMO-1 primarily on Lys21 (Desterro *et al.*, 1998), which is utilized for ubiquitination too. Thus, SUMO-1-modified IκBa can not be ubiquitinated and is resistant to proteasome-mediated degradation. As a result, over-expression of SUMO-1 inhibits signal-induced activation of NF-κB-dependent transcription.

Mdm2 is a *bona fide* RING finger E3 (Fang *et al.*, 2000; Honda and Yasuda, 2000). It has been implicated in the proteasome-dependent degradation of tumor suppressor protein p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997) via targeting p53 ubiquitination (Fuchs *et al.*, 1998; Honda *et al.*, 1997). SUMO modification of Mdm2 takes place on a lysine residue at position 446, which is the primary site for Ub

conjugation to Mdm2. Thus, upon its sumoylation, Mdm2 is no longer subject to ubiquitination, gaining stability and greater E3 activity toward its substrate, p53 (Fuchs *et al.*, 1998; Honda *et al.*, 1997).

1.2 Post-replication repair

DNA carries the genetic information of an organism. A minimal change in DNA may have lethal effect on the organism. The fact that DNA is constantly damaged by exogenous and endogenous agents made prokaryotic and eukaryotic cells evolve a variety of mechanisms to remove DNA lesions. In yeast and higher eukaryotes, DNA damage repair is executed mainly by three pathways (Prakash *et al.*, 1993). **N**ucleotide **e**xcision **r**epair (NER) is responsible for recognizing and removing UV-induced pyrimidine dimmers and other damage or chemically modified bases and nucleotides. Double-strand breaks are repaired via **h**omologous **r**ecombination (HR). Finally, the third system is called **p**ost-**r**eplication **r**epair (PRR). It is activated during DNA synthesis to bypass DNA lesions which cause a stalling of the replication machinery or after DNA synthesis to fill in single-stranded gaps which were produced in the newly synthesized strand due to the inability of replication machinery bypassing the damaged sites. While NER and HR are mechanistically quite well understood, little is known about the molecular details of PRR. However, genetic studies have discovered many genes which are involved in PRR.

1.2.1 *RAD6* and *RAD18*

PRR in *S. cerevisiae* is dependent on *RAD6* and *RAD18*. *RAD6* itself encodes an E2 (Jentsch *et al.*, 1987) which has a 149 amino acid core domain common to other E2s and a 23-residue C-terminal "tail" comprising almost entirely of acidic residues (Morrison *et al.*, 1988). RAD6 is epistatic to all other members of this repair pathway. Mutations in this gene confer a pleiotropic phenotype, involving a high degree of sensitivity towards DNA-damaging agents, a defect in damage-induced mutagenesis as well as an increased rate of spontaneous mutagenesis, but also defects unrelated to DNA repair, including a reduced growth rate, temperature sensitivity and defective sporulation (Prakash, 1994). All cellular functions of Rad6 require its E2 activity, since substitution of the active site cysteine (Cys88) residue confers the *rad6*Δ phenotypes (Sung *et al.*, 1990). Rad6 functions as an E2 through interaction with at least three RING finger proteins, Ubr1, Bre1, and Rad18. Ubr1/Rad6 is involved in N-end rule protein degradation (Dohmen *et al.*, 1991), in which the *in vivo* half-life of some proteins is determined by the nature of their N-terminal amino acids (Bachmair *et al.*, 1986). Bre1/Rad6 functions in mono-ubiquitination of histone H2B (Joazeiro and Weissman, 2000), leading to chromatin remodeling. Rad18/Rad6 plays the key role in PRR. Unlike Rad6, Rad18, which contains the aforementioned RING finger motif and has ATPase and ssDNA binding activities (Bailly *et al.*, 1997a), is only involved in PRR. Mutants of *rad18* resemble those of *rad6* in their high degree of sensitivity to a wide range of DNA damaging agents and defects in PRR activity (di Caprio and Cox, 1981; Prakash, 1981).

1.2.2 Other genes involved in PRR

Other genes that belong to the *RAD6* epistasis group include *MMS2*, *UBC13*, *RAD5*, *RAD30*, *REV1*, *REV3*, *REV7*, and *POL30* (Barbour and Xiao, 2003; Broomfield *et al.*, 2001). The *rad6* and *rad18* mutations are epistatic to mutation in each of these genes.

1.2.2.1 *MMS2*, *UBC13* and *RAD5*

The *MMS2* gene was isolated from a **m**ethyl **m**ethane**s**ulfonate (MMS) sensitive mutant (Broomfield *et al.*, 1998). Mms2 is a **U**bc (Ub conjugating enzyme) **e**nzyme **v**ariant (Uev), which is similar in sequence to Ubcs but lacks Ub conjugation activity because of the absence of an active-site cysteine residue. Mms2 forms a stable complex with Ubc13, a canonical E2 (Brown *et al.*, 2002; Hofmann and Pickart, 1999). Ubc13 catalyzes the formation of poly-Ub chain via Lys63 (Hofmann and Pickart, 1999), instead of the conventional Lys48 chain assembly. Mms2 plays a regulation role in the formation of this atypical poly-Ub chain by orienting the acceptor Ub through noncovalent contacts so that its Lys63 is available to the donor Ub bound to Ubc13 (McKenna *et al.*, 2001; Pastushok and Xiao, 2004). Rad5 is a RING finger protein and has Swi2/Snf2 homologous domains (Johnson *et al.*, 1992; Johnson *et al.*, 1994; Richmond and Peterson, 1996). It has ssDNA-dependent ATPase activity but no helicase activity (Johnson *et al.*, 1994). Rad5 interacts with Ubc13 by means of its RING finger domain (Ulrich, 2003; Ulrich and Jentsch, 2000), suggesting Rad5 is an E3 for Ubc13-Mms2 dependent poly-Ub conjugation.

1.2.2.2 ***RAD30***

RAD30 encodes DNA polymerase η (Polη) (Johnson *et al.*, 1999b), a member of Y-family polymerases. The amino acid sequence of Y-family polymerases is unrelated to that of classical DNA polymerases and is characterized by five conserved motifs, I-V (Ohmori *et al.*, 2001). Polη is proficient to replicate through a *cis-syn* thymine-thymine (TT) dimer, inserting an A opposite the 3'-T and 5'-T of the TT dimer (Johnson *et al.*, 1999b). Polη also functions in the error-free bypass of UV-induced (6-4) photoproducts (Yu *et al.*, 2001) and the oxidative lesion 7,8-dihydro-8-oxoguanine (Haracska *et al.*, 2000b). Polη replicates through other DNA lesions inefficiently in either error-free or error-prone pathways (Haracska *et al.*, 2000a; Levine *et al.*, 2001; Minko *et al.*, 2001). Mutations in Polη in humans cause a cancer-prone syndrome, the variant form of xeroderma pigmentosum (XP-V) (Johnson *et al.*, 1999a; Masutani *et al.*, 1999). Cells from XP-V individuals are deficient in the replication of UV-damaged DNA (Cordeiro-Stone *et al.*, 1997; Lehman *et al.*, 1975).

1.2.2.3 ***REV* genes**

REV genes were originally identified as genes responsible for reversions of UV-induced mutations in *S. cerevisiae*. Rev1 is a deoxycytidyl transferase, being able to transfer a single dCMP to the 3' end of a DNA primer in a template-dependent reaction (Nelson *et al.*, 1996a). It is also a member of Y-family DNA polymerases. Rev3 is a 173 kDa protein with conserved DNA polymerase motifs (Morrison *et al.*, 1989). It binds to

Rev7 to form dimeric DNA polymerase ζ (Polζ (Nelson *et al.*, 1996b). Polζ can replicate through *cis-syn* TT dimer, although it does so rather inefficiently (Nelson *et al.*, 1996b). Polζ is highly inefficient at inserting nucleotides opposite DNA lesions, but it is efficient at extending from mismatched bases inserted by Rev1, Polη, Polδ, or Polι (Haracska *et al.*, 2001; Johnson *et al.*, 2001; Johnson *et al.*, 2000). A Recent study showed that **p**roliferating **c**ell **n**uclear **a**ntigen (PCNA) can stimulate Polζ to bypass UV-induced DNA damage site (Garg *et al.*, 2005). Extension by Polζ from a Polη misinsertion opposite the 3' nucleotide of a T-T (6-4) UV photoadduct (Haracska *et al.*, 2001; Johnson *et al.*, 2001; Johnson *et al.*, 2000), necessarily requires insertion opposite the 5'-thymine of the lesion by Polζ, indicating that this enzyme is capable of inserting nucleotides opposite a lesion. Polζ is likely to be responsible for inserting nucleotides opposite some DNA lesions *in vivo* (Gibbs *et al.*, 2005). In mammalian cells, Rev1 may work with Polζ or without Polζ in specific DNA lesion bypass. For example, Rev1 is involved in **h**omologous **r**ecombination (HR)-mediated repair of **d**ouble-**s**trand **b**reaks (DSBs) as a component of Polζ complex and also participates in *Ig* gene conversion without interacting with Polζ (Okada *et al.*, 2005).

1.2.2.4 *POL30*

PCNA, encoded by *POL30*, is the eukaryotic sliding clamp required for processive DNA synthesis. The crystal structure of yeast PCNA shows that a homotrimer of PCNA forms a closed ring to encircle double-stranded DNA and to load onto it by

replication **f**actor **C** (RF-C) in an ATP-dependent reaction (Lee and Hurwitz, 1990; Tsurimoto and Stillman, 1991). PCNA directly associates with various DNA polymerases and functions as a sliding clamp, thereby stimulating accurate and processive DNA synthesis (Bambara *et al.*, 1997; Kelman and Hurwitz, 1998).

1.2.2.4.1 PCNA ubiquitination and sumoylation

Recently, PCNA was found to be covalently modified by either Ub or SUMO (Hoege *et al.*, 2002). *S. cerevisiae* PCNA could be monosumoylated at Lys127 or Lys164. The prominent site in PCNA for SUMO conjugation is Lys164, which is conserved within eukaryotes. SUMO conjugation to PCNA occurs in normal cells. PCNA is also modified by Ub. Unlike SUMO, one to more than four Ub moieties can be conjugated to PCNA, but only after cells were treated with a sublethal dose of DNA-damaging agents. The site of Ub attachment is identical to that of SUMO at Lys164. Further studies revealed that ubiquitination of PCNA were completely abolished in *rad6* mutants, whereas sumoylation was unaffected; mutants in *UBC13*, *MMS2*, or *RAD5* led to the disappearance of poly-Ub conjugates, but mono-Ub modified PCNA remained. These results indicated that monoubiquitination of PCNA is performed by the Rad6-Rad18 complex, whereas polyubiquitination is catalyzed by Ubc13-Mms2-Rad5. This unique activity of Ubc13 conjugates a poly-Ub chain of PCNA through Lys63.

1.2.2.4.2 PCNA's regulatory role in PRR

PRR has been suggested to replicate through DNA lesions via three different pathways: the Polζ- and Polη-dependent **t**rans**l**esion DNA **s**ynthesis (TLS) and Rad5-Mms2-Ubc13-dependent damage-avoidance pathway (Broomfield *et al.*, 2001; Torres-Ramos *et al.*, 2002). The Rad5-Mms2-Ubc13 dependent pathway is believed to involve a recombination replication process using the newly synthesized daughter strand of the sister duplex as a template (Li *et al.*, 2002; Torres-Ramos *et al.*, 2002). Since PCNA is involved in PRR (Torres-Ramos *et al.*, 1996) and it can be mono- or poly-Ub conjugated by other PRR members, it is attractive to speculate that PCNA modification plays a regulatory role in switching among different PRR pathways (Fig. 1-7). A recent study indicated that mono-ubiquitination of PCNA leads to Polζ- and Polη-dependent TLS (Stelter and Ulrich, 2003). Rad18 interacts with Polη suggesting Polη is recruited to replication stalling sites by Rad18, and Polη preferentially binds to monoubiquitinated PCNA both *in vitro* and *in vivo* (Watanabe *et al.*, 2004), which support the role of mono-Ub conjugate of PCNA in TLS. It was suggested that mono-ubiquitination of PCNA disrupts the replication machinery so that replicative polymerase dissociates and TLS polymerase binds PCNA to bypass DNA lesion. PCNA polyubiquitination stimulates the damage-avoidance pathway which involves a recombination replication process (Stelter and Ulrich, 2003). The effects of PCNA SUMO modification seem elusive. Since *rad6* and *rad18* mutants are more sensitive to UV than the *pol30-119* (lysine 164 residue mutated to arginine) mutant which inactivates whole PRR system, it is speculated that K164R mutation activates an alternate repair pathway. The very similar UV sensitivities

of *pol30-119 rad52* and *rad6 rad52* double mutants suggested this pathway is Rad52-dependent recombination (Haracska *et al.*, 2004; Pfander *et al.*, 2005). In normal cells, PCNA is prevalently modified by SUMO during S phase (Hoege *et al.*, 2002). This modification is suggested to inhibit the Rad52-dependent recombinational pathway (Haracska *et al.*, 2004; Papouli *et al.*, 2005; Pfander *et al.*, 2005).

1.2.3 Roles of *SRS2* in PRR

Srs2 is linked to PRR, as mutations in *SRS2* suppress the UV sensitivity of *rad6* and *rad18* mutants (Aboussekhra *et al.*, 1989; Lawrence and Christensen, 1979; Schiestl *et al.*, 1990b). Srs2 protein has 3' to 5' DNA helicase and DNA-dependent ATPase activities (Rong and Klein, 1993). The suppression effect is due to the activation of recombination by *SRS2* deletion (Schiestl *et al.*, 1990a; Schiestl *et al.*, 1990b). Recent studies showed that DNA strand exchange mediated by Rad51 is inhibited by Srs2. In Rad51-dependent recombination, Rad51 is loaded on ssDNA to form a nucleoprotein filament which is capable of interacting with a second DNA molecule, thus initiating strand exchange (Krogh and Symington, 2004). Srs2 disrupts Rad51 presynaptic filament and inhibits the recombination (Krejci *et al.*, 2003; Veaute *et al.*, 2003). The inhibitory function of PCNA sumoylation on the *RAD52* recombinational pathway is similar to that of Srs2 protein. The potential links between PCNA sumoylation and Srs2 were studied. The interaction between Srs2 and PCNA was discovered in yeast two-hybrid assays and

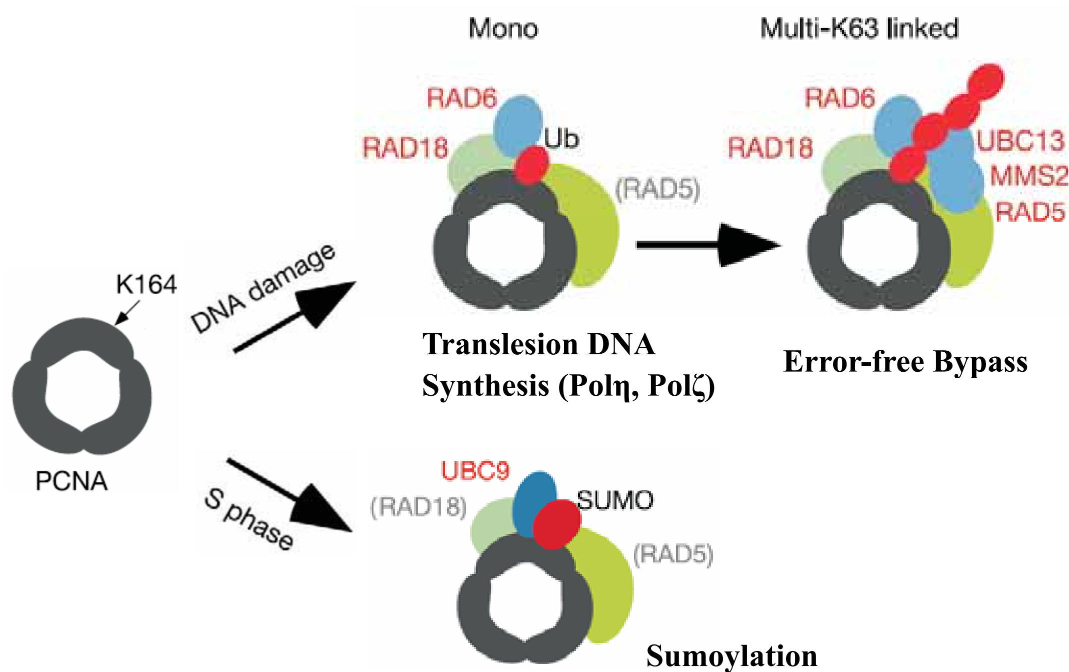


Fig. 1-7 Proposed model for PRR in *S. cerevisiae*. Mono-ubiquitination of PCNA at Lys164 is dependent on Rad6 and Rad18, while Rad5, Mms2 and Ubc13 are required to attach additional ubiquitin to the conjugate through a Lys63 chain assembly. Mono-ubiquitination of PCNA leads to translesion DNA synthesis, while poly-ubiquitination of PCNA shuttles the repair into an error-free bypass pathway. In the absence of DNA-damaging agents, PCNA is modified by SUMO during S phase.

pull-down experiments; this interaction is strongly augmented by PCNA sumoylation (Haracska *et al.*, 2004; Papouli *et al.*, 2005; Pfander *et al.*, 2005). The *rad18srs2*, *rad18siz1*, *rad18srs2siz1* and *rad18srs2pol30-K127/164R* mutants all showed nearly identical phenotypes. This epistatic relationship reveals that PCNA sumoylation and Srs2 function in the same pathway. PCNA sumoylation, which occurs even in the absence of DNA damage, seems to be a guarding mechanism preventing unwanted recombination during S phase. This inhibitory function is achieved though recruiting Srs2 by sumoylated PCNA to replication forks to prevent formation of Rad51 presynaptic filament.

1.2.4 Some human PRR genes

Much less is known about PRR in humans. The homologs of yeast Rad6 have been identified in humans (Koken et al., 1991). Interestingly, the *RAD6* homologous gene in human is duplicated, in contrast to yeast where *RAD6* is a single copy gene. The two proteins HHR6A (human homolog of Rad6) and HHR6B, which are 95% identical in amino acid sequence, share 69% sequence identity with *S. cerevisiae* Rad6 (Fig. 1-8). The *S. cerevisiae* Rad6 has a 23-residue acidic tail at the C-terminus. Mutational analysis has shown the acidic domain to be essential for sporulation (Morrison et al., 1988). Neither of human Rad6 homologs possesses this domain. Genetic complementation experiments revealed that HHR6A/B can carry out the DNA repair and mutagenesis functions of Rad6, but confer only a very low level of sporulation ability in *S. cerevisiae*

*rad6*Δ mutants (Koken et al., 1991). The structure of HHR6B showed no significant difference from that of *S. cerevisiae* Rad6 (Miura et al., 2002).

In human, only a single homolog of *RAD18* has been identified (Tateishi *et al.*, 2000; Xin *et al.*, 2000). Human Rad18 (hRad18), a 495 aa protein, shares 26% identical and 59% similar residues with yeast Rad18. Furthermore, several structure features of yeast Rad18 are also present in hRad18, including an N-terminal RING finger domain, and a C2HC zinc finger motif. *RAD18* is dispensable for cell viability, since *Rad18*$^{-/-}$ mouse embryonic cells exhibited a similar growth rate to that of wild type cells. However, *Rad18* knockout mouse embryonic stem cells and chicken DT40 cells showed increased sensitivity to multiple types of DNA damaging agents and enhanced genomic instability as determined by increased sister-chromatid exchange (Tateishi et al., 2003; Yamashita et al., 2002). These observations demonstrate that *RAD18* is indeed involved in PRR in vertebrate cells as well as in yeast.

The human *UBC9* (*hUBC9*) gene encodes a 17-kDa protein having 56% aa sequence identity with yeast Ubc9. Human Ubc9 can fully complement a yeast strain that has a temperature-sensitive yeast *ubc9* gene mutation to fully restore normal growth (Wang et al., 1996). hUbc9 shares 41% sequence identity with HHR6B (Fig. 1-8). Although the structure of hUbc9 is very similar to the structures of known Ubc enzymes, it still shows important differences. Compared with the structures of HHR6B and *S. cerevisiae* Rad6, hUbc9 has two insertions in the Ubc core domain. The first insertion occurs at residues 32–36 and these 5 residues form most of a very exposed β-hairpin that

```
hUbc9     MSGIALSRLAQERKAWRKDHPFGFVAVPTKNPDGTMNLMNWECAIPGKKGTPWEGGLFKL
yRad6     MSTPARRRLMRDFKRMKEDAPPGVSASPLPD-----NVMVWNAMIIGPADTPYEDGTFRL
HHR6A     MSTPARRRLMRDFKRLQEDPPAGVSGAPSEN-----NIMVWNAVIFGPEGTPFEDGTFKL
HHR6B     MSTPARRRLMRDFKRLQEDPPVGVSGAPSEN-----NIMQWNAVIFGPEGTPFEDGTFKL

hUbc9     RMLFKDDYPSSPPKCKFEPPLFHPNVYPSGTVCLSILEEDKDWRPAITIKQILLGIQELL
yRad6     LLEFDEEYPNKPPHVKFLSEMFHPNVYANGEICLDILQNR--WTPTYDVASILTSIQSLF
HHR6A     TIEFTEEYPNKPPTVRFVSKMFHPNVYADGSICLDILQNR--WSPTYDVSSILTSIQSLL
HHR6B     VIEFSEEYPNKPPTVRFLSKMFHPNVYADGSICLDILQNR--WSPTYDVSSILTSIQSLL

hUbc9     NEPNIQDPAQAEAYTIYCQNRVEYEKRVRAQAKKFAPS---------------------
yRad6     NDPNPASPANVEAATLFKDHKSQYVKRVKETVEKSWEDDMDDMDDDDDDDDDDDDDEAD
HHR6A     DEPNPNSPANSQAAQLYQENKREYEKRVSAIVEQSWRDC--------------------
HHR6B     DEPNPNSPANSQAAQLYQENKREYEKRVSAIVEQSWNDS--------------------
```

Fig. 1-8 Protein sequence comparison of human Ubc9 (hUbc9), human Rad6 (HHR6A and HHR6B) and *S. cerevisiae* Rad6 (yRad6).

connects strand β1 and β2. The second insertion occurs at residues 100–101, and forms a bulge in a loop (residues 94–102) (Tong et al., 1997). It appears that such insertions can provide additional binding site for substrates.

1.3 Hypothesis

PCNA can be modified by Ub and SUMO at the same site. SUMO modification of PCNA, which is prevalent during the S phase, might be a device used for keeping Rad52 recombinant pathway in check during the S phase. PCNA ubiquitination is a prerequisite for Rad6-Rad18 dependent lesion bypass processes. The molecular mechanisms that regulate the balance between sumoylation and ubiquitination of PCNA remain unclear. When DNA damage occurs, Rad18 first binds to ssDNA and recruits Rad6 to the damaged site where they mono-ubiquitinate PCNA. Thus Rad18 is possibly involved in regulation of the switch from sumoylation to ubiquitination of PCNA. There are two possibilities how Rad18 is involved in the regulation. Firstly, although Rad18 is not an E3 for sumoylation, it participates in the process of PCNA SUMO modification by interacting with sumoylation protein(s). Once DNA is damaged, Rad18 switches to PCNA ubiquitination by forming complex with Rad6. Secondly, Rad18 has no function in PCNA sumoylation, instead it interferes with this modification by interacting with a sumoylation protein and keeping it away from PCNA. Studying the interactions of Rad18 with Rad6 and Ubc9, and finding out the domains of Rad18 required in the interactions can help to understand whether and how Rad18 regulates the switch from sumoylation to

ubiquitination of PCNA.

1.4 Objectives of this study

To better understand the mechanisms of PRR, this study aims to define domains involved in the formation of several protein complexes which play key roles in PRR. The special aims of this study are:

(1) to map the domain of hRad18 interacting with HHR6B

(2) to study the interaction between hRad18 and hUbc9, and map the domain(s) of hRad18 required for the interaction

CHAPTER TWO

MATERIALS AND METHODS

2.1 Yeast Genetics

2.1.1 Yeast strains and cell cultures

The *S. cerevisiae* strains used in this thesis are Y190 (*MATa*, *gal4-542*, *gal80-538*, *his3*, *trp1-901*, *ade2-101*, *ura3-52*, *leu2-3, 112*, URA3::GAL1-lacZ, Lys2::GAL1-HIS3) and PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4delta gal80delta GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*).

Yeast cells were cultured at 30°C either in a rich YPD medium or in a synthetic dextrose (SD) medium. YPD is a standard, complex medium composed of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. SD medium is used for selective growth of yeast auxotrophs. It contains 0.67% yeast nitrogen base without amino acids (YNB), 2% glucose, and addition of any auxotrophic supplements needed. The auxotrophic supplements include 30 mg/L L-isoleucine, 150 mg/L L-valine, 20 mg/L adenine hemisulfate salt, 20 mg/L arginine HCl, 20 mg/L L-histine HCl monohydrate, 100 mg/L L-leucine, 30 mg/L lysine HCl, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 200 mg/L L-threonine, 20 mg/L L-tryptophan, 30 mg/L L-tyrosine, 20 mg/L uracil. To make a selection medium, all of the above auxotrophic supplements except the one(s) that can be synthesized by the selection gene on the plasmid were added to YNB media. The auxotrophic supplements were made in 100x stocks and added into

media prior to autoclaving. To make plates, 2% agar was added to either YPD or SD medium prior to autoclaving.

Yeast cells can be stored for up to four months on plates sealed with parafilm at 4°C. For long term storage, yeast cells were grown in appropriate liquid medium (rich or minimal media) at 30°C overnight. 0.7 ml of the culture was added into 0.3 ml 50% sterile glycerol and then stored at -70°C.

2.1.2 Yeast transformation

Yeast cells were transformed using a dimethyl sulfoxide (DMSO)-enhanced method as described (Hill *et al.*, 1991). 2 ml of yeast cells was grown overnight at 30°C in rich media (or appropriate minimal media), and subcultured into 3 ml of fresh media. When the yeast cells reached a mid-logarithmic phase of growth, they were pelleted by centrifugation. The yeast cells were washed in 400 µl LiOAc solution [0.1 M lithium acetate, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA], and resuspended in 100 µl of the same solution. 5 µl of denatured carrier DNA (single strand salmon sperm DNA) and 1-5 µl of transforming DNA were added and mixed well. After incubation at room temperature for 5 minutes, 280 µl of PEG4000 (50% polyethylene glycol 4000 in LiOAc solution) was added and mixed by inverting the tube 4-6 times. After the transformation mixture was incubated for 45 minutes at 30°C, 39 µl of DMSO was added, followed by a 5-minute heat shock in a 42°C waterbath. Yeast cells were then washed with sterile double distilled

water (ddH_2O) and resuspended in 100 µl of ddH_2O. The resuspended cells were plated on the appropriate minimal media.

2.1.3 *In vivo* assay of protein interaction using yeast two-hybrid system

2.1.3.1 β-gal activity assay

Yeast two-hybrid strain Y190 was transformed simultaneously with different combinations of pGBT and pGAD constructs. A filter assay was employed to determine the β-galactosidase (β-gal) activity (Bartel and Fields, 1995). For each combination, at least 3 independent co-transformants were resuspended in sterile ddH_2O at equal densities, spotted onto SD-Leu-Trp plates and allowed to grow for 3 days. Cells were transferred to a Whatman No.1 filter paper, immersed in liquid nitrogen for 10 seconds to permeablize cells, and placed on top of another filter which was presoaked with a mixture of 1.8 ml Z-buffer (16.10 g/L $Na_2HPO_4.7H_2O$, 5.50 g/L $NaH_2PO_4.\ H_2O$, 0.75 g/L KCl and 0.246 g/L $MgSO4.7H_2O$, pH 7.0) containing 5 µl β-mercaptoethanol and 45 µl of 20 mg/ml X-gal dissolved in *N,N*-dimethylformamide. Plates were sealed with parafilm and incubated at 30°C. Color development was monitored during the 8 hours of incubation. Colonies which turned blue during 8-hour incubation were considered positive and indicated as +, Colonies remaining unchanged after 8-hour incubation were considered negative and indicated as -.

2.1.3.2 *HIS3* reporter gene assay

Yeast two-hybrid strain PJ69-4A was transformed simultaneously with different combinations of pGBT and pGAD constructs. For each combination, at least 3 independent co-transformants were cultured in SD-Leu-Trp media and allowed to grow overnight. The next day, 5-10 µl of each co-transformant was spotted on SD-Leu-Trp-His plates plus different concentration of 3-AT. The plates were incubated at 30°C for 2-4 days to test the activation of the *HIS3* gene.

2.2 Molecular Biology and Biochemistry Techniques

2.2.1 Bacterial culture and storage

The *E. coli* strain DH10B (GibcoBRL, Grand Island, NY, USA) was used for bacterial transformation. Transformed cells were cultured in LB liquid or agar media (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl and 1.25% agar for plates) containing specific antibiotic in an appropriate concentration. For short-term storage (2-3 months), transformed cells were stored on LB plates containing antibiotic. For long-term storage, transformed cells were grown overnight in 900 µl of LB and immediately placed in a -70°C freezer after mixing with 100 µl of DMSO.

2.2.2 Preparation of competent cells

E. coli competent cells for electroporation were prepared as suggested in the BioRad *E. coli* Pulser manual. One liter of culture was incubated until an OD_{600nm} of 0.6 was reached. The culture was centrifuged at 3500 rpm for 5 minutes in a Beckman GSA

rotor and the pellet was resuspended in 500 ml of 10% ice-cold sterile glycerol. The centrifugation was repeated 4 times, with each pellet resuspended in a reduced volume; the last pellet was resuspended in 4 ml ice-cold, sterile 10% glycerol. The cells were aliquoted into 1.5 ml eppendorf tubes to a volume of 25 µl, and were quickly placed in the -70°C freezer for storage.

2.2.3 Bacterial transformation

All bacterial transformations in this study were carried out by the electroporation method. The DNA to be transformed was added to *E. coli* competent cells and the cell mixture was transferred to a pre-chilled electroporation cuvette (BioRad). After a brief incubation on ice, the cells were exposed to a voltage of 1.8 kV (for cuvettes with 0.1 mm width) using the *E. coli* Pulser (BioRad). 400 µl of SOC medium was added to the cuvette after electroporation. The cells were transferred to a 1.5 ml eppendorf tube, incubated at 37°C for half an hour, then spreaded on LB plates containing appropriate antibiotic and incubated at 37°C overnight.

2.2.4 Rapid preparation of plasmid DNA

2.2.4.1 Quick DNA isolation

Plasmid amplification and isolation was performed following the methods described by Maniatis *et al.* (1982). Single colonies were inoculated into 2 ml LB liquid media containing appropriate antibiotic and grown overnight at 37°C. Cells were

collected by centrifugation and the pellet was resuspended in 350 µl of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl, pH 8.0). After mixing with 20 µl of lysozyme (10 mg/ml; Sigma, St Louis MI), the mixture was quickly placed in a boiling water-bath for 40 seconds, followed by centrifugation for 10 minutes at top speed. The pellet was removed with a toothpick, and 8 µl of 5 M NaCl and 2 volumes of ethanol were added to precipitate the DNA. Precipitated DNA was dried briefly in a vacuum device, and redissolved in 30-50 µl ddH_2O.

2.2.4.2 DNA isolation with commercial miniprep kit

The kit used is the Quantum Prep® Plasmid Miniprep Kit (Bio-Rad). Its application protocols are summarized below and the reagents mentioned in this method were provided within the kit.

Overnight cultures (3-5 ml) of individual colonies were pelleted by centrifugation and resuspended in 200 µl of Cell Resuspension Solution. 250 µl of the Cell Lysis Solution were added and mixed gently until the solution became viscous and slightly clear. Then, 250 µl of the Neutralization Solution were added and mixed gently until a visible precipitate formed. The solution mixture was centrifuged at 12,000-14,000 x *g* for 5 minutes and the supernatant was treated with 200 µl of Quantum Prep matrix suspension by pipetting up and down. Then the DNA containing matrix suspension (*ca.* 800 µl) was transferred to a Spin Filter cap that was loaded on a decapped 1.5-ml microcentrifuge tube. The matrix gel was pelleted in a Spin Filter cap by centrifugation

30 seconds at the maximum speed, washed twice by adding 500 µl of Wash Solution into the Spin Filter cap and re-centrifuged. The last wash was followed by an additional centrifugation to remove the residual ethanol. The plasmid DNA was eluted with 100 µl of distilled water or TE buffer added to the matrix gel in the cap, and the cap was transferred onto a fresh collection tube and centrifuged for 30 seconds. The plasmid DNA isolated can be used for direct DNA sequencing, cell transformation, enzymatic digestion and modification.

2.2.5 Restriction endonuclease digestion

Restriction endonuclease (GibcoBRL, Novagen, and New England Biolabs) digestions were routinely performed to prepare the gene constructs for ligation into vectors, to cleave circular vector DNA for fragment insertion, or to screen individual vectors clones for the presence of fragment insert. The procedure was basically as described by Sambrook *et al.* (1989). In total volume of 10 to 50 µl, microgram quantities of DNA were cut with 5 to 25 U restriction enzyme in the reaction buffer recommended by the supplier. Double digestion was performed whenever both enzymes functioned well in the same reaction buffer. If not, the DNA was digested with the enzyme requiring the lower salt concentration, followed by the second enzyme required additional salt without recovering the DNA between reactions. The reactions were usually carried out at 37°C for 60 to 120 minutes before further treatment or analysis.

2.2.6 Agarose gel electrophoresis and DNA fragment isolation

For analysis of plasmid DNA, 0.7% agarose gels were used in this study. Gels were run in 1X TAE buffer (40 mM Tris-acetate, 2 mM Na_2EDTA) and stained in 0.5 μg/ml ethidium bromide (EtBr). DNA was visualized under long-wave UV light after destaining in water for 10 minutes.

The method of DNA fragment isolation from agarose gels was adapted from Wang and Rossman (Wang and Rossman, 1994). After enzyme digestion, the sample was electrophoresed through 0.7% agarose gel and stained with EtBr. The band of interest was identified using an UV-illuminator and cut out of the gel. A 0.5 ml microcentrifuge tube was pierced at the bottom, and packed with chopped cheesecloth. The gel slice containing the DNA fragment was placed into the prepared tube, which was inserted into another 1.5 ml tube, left it in the -70°C freezer for 20 minutes and centrifuged for 10 minutes at top speed. The DNA eluted was purified by phenol/chloroform (1:1) extraction as below **2.2.7**.

2.2.7 Phenol/chloroform Extraction of DNA

Phenol/chloroform extraction was used to remove proteins from nucleic acid samples. The nucleic acid sample was diluted to about 400 μl in a 1.5 ml eppendorf tube. Equal volumes (*ca.* 200 μl) of Tris buffered-phenol and chloroform-isoamyl alcohol mixture (previously mixed 24:1) were added to the tube, the tube was then inverted several times to mix the phases. After centrifugation at 13,000 x *g* for 3 minutes, the

upper layer was transferred to a new tube, discarding the tube with bottom layer. To remove any trace phenol, 400 µl chloroform-isoamyl alcohol mixture was added to the new tube and the phases was mixed by inverting. The upper layer was transferred to a new tube after spinning. To precipitate the DNA, 10 ul of 5 M NaCl and two times the volume of cold ethanol (800 ul) were added and the tube was placed at -20°C for at least 30 minutes. After centrifugation at 13,000 x *g* for 10 minutes, the supernatant was discarded. The DNA sample in the tube was dried in a vacuum device and resuspended in ddH_2O.

2.2.8 Phosphatase treatment of vector DNA

When the fragment DNA was inserted into vector DNA cleaved with only a single restriction enzyme, the likelihood of self-ligation of the vector was minimized by dephosphorylation with phosphatase treatment. Vector DNA (usually less than 1 µg) was digested as usual in a 40 µl volume. After digestion, the reaction mixture was further supplemented with 1 µl of calf intestinal phophatase (1 U) and incubated at 37°C for about 20 minutes. DNA then was recovered as above **2.2.7**.

2.2.9 Ligation of fragment into plasmid vector

Ligation was done as described by Sambrook *et al.* (1989). Plasmid DNA was linearized with the appropriate restriction enzyme(s) and purified. Purified DNA fragment (as an insert) was combined with linearized vector DNA in a 20 µl volume with

50 mM Tris-HCl, pH7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 5 U T4 DNA ligase. To get a high chance of concatermeric ligation and compromise the chance of vector DNA self-ligation, the molar ratio of vector DNA to insert DNA was set to ~1:3. The mixture was incubated at 16°C overnight. This reaction was used to transform *E. coli* competent cells.

2.2.10 Plasmids used in this study

All plasmids used or constructed in this study are listed in **Table 2-2**.

2.2.10.1 Plasmids for yeast two-hybrid assays

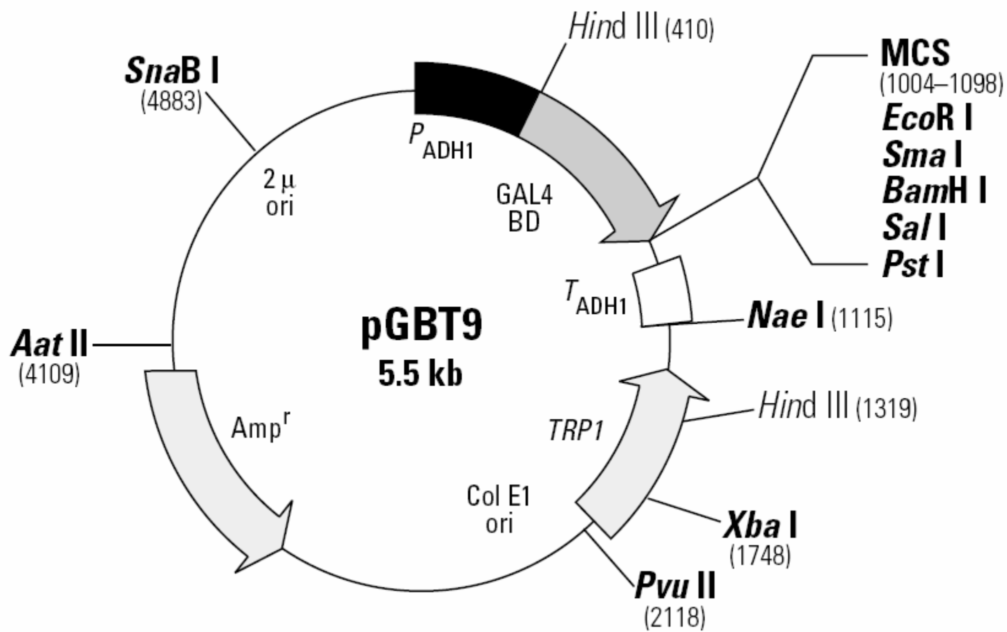
In this system, two different sets of vectors were employed to assess protein-protein interactions. One set of vectors contains the Gal4 DNA-binding domain (BD), such as pGBT9 and its derivative pGBT9-Bg (Fig. 2-1). Another set of vectors contains Gal4 DNA-activation domain (AD), namely pGAD424 and its derivative pGAD424-Bg (Fig. 2-2).

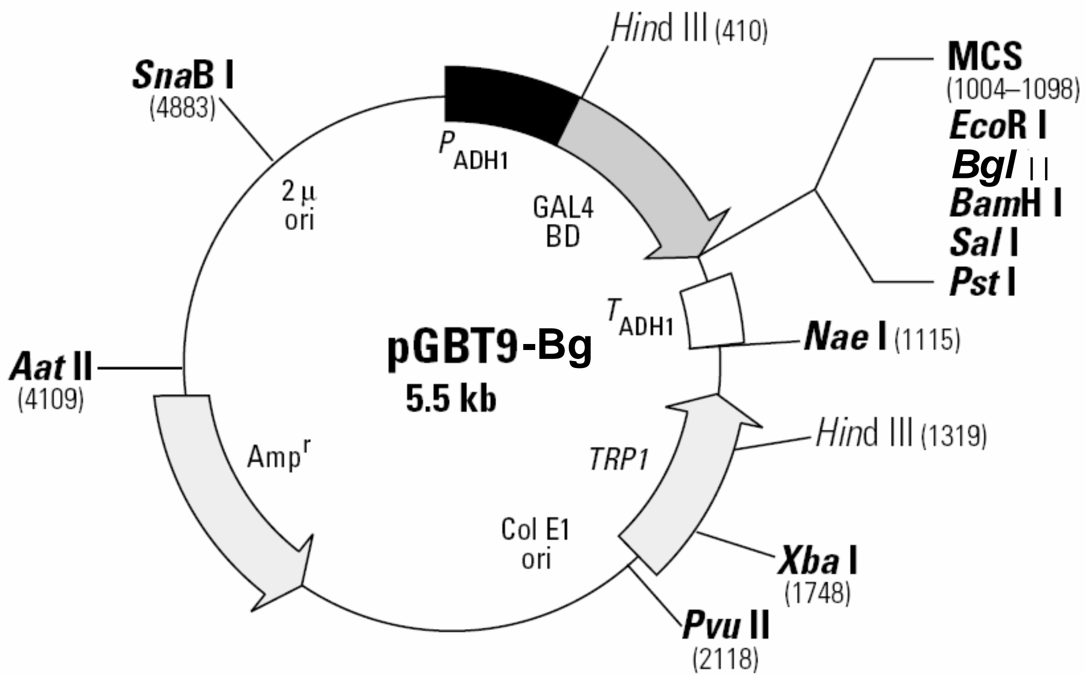
The yeast *UBC9* gene fragment was released from pYES-UBC9 by *Bam*HI and *Xho*I double digestion. This fragment was cloned into *Bam*HI and *Sal*I sites of pGBT9-Bg and pGAD424-Bg vectors to produce pGBT-UBC9 and pGAD-UBC9, respectively. The plasmids pGBT-hUBC9 and pGAD-hUBC9 were received from Dr. Z. Shen (U of New Mexico).

Table 2-2 plasmid constructs

Plasmid	**Relevant characteristics**	**Source**
pYES-UBC9	Full length yeast *UBC9* in pYES2.0	
pGBT9	Yeast two-hybrid vector	Clontech
pGBT9-Bg	Yeast two-hybrid vector	W. Xiao
pGAD424	Yeast two-hybrid vector	Clontech
pGAD424-Bg	Yeast two-hybrid vector	W. Xiao
pGBT-UBC9	Full length yeast *UBC9* in pGBT9-Bg	This study
pGAD-UBC9	Full length yeast *UBC9* in pGAD424-Bg	This study
pGBT-hUBC9	Full length human *UBC9* in pGBT9	Z. Shen
pGAD-hUBC9	Full length human *UBC9* in pGAD424	Z. Shen
pGBT-RAD6	Full length yeast *RAD6* in pGBT9	This study
pGAD-RAD6	Full length yeast *RAD6* in pGAD424	This study
pACT-HHR6A	Full length *HHR6A* in pACTII	This study
pGAD-HHR6B	Full length *HHR6B* in pGAD424	This study
pGBT-RAD18	Full length yeast *RAD18* in pGBT9	This study
pGAD-RAD18	Full length yeast *RAD18* in pGAD424	This study

pGBT-RAD18-40	yeast *rad18* (aa 371-410) in pGBT9	This study
pGAD-RAD18-40	yeast *rad18* (aa 371-410) in pGAD424	This study
pGBT-hRAD18	Full length human *RAD18* in pGBT9-Bg	This study
pGBT-hRAD18N	human *rad18* (aa 1-93) in pGBT9-Bg	This study
pGAD-hRAD18N	human *rad18* (aa 1-93) in pGBT9-Bg	This study
pGBT-hRAD18C	human *rad18* (aa 94-495) in pGBT9-Bg	This study
pGBT-myc hRAD18	Full length human *RAD18* with N-terminal myc tag in pGBT9	M. Yamaizumi
pGBT-hRAD18C28F	human *rad18* (Cys28 mutated to Phe) with C-terminal myc tag in pGBT9	M. Yamaizumi
pGBT-hRAD18C207F	human *rad18* (Cys207 mutated to Phe) with C-terminal myc tag in pGBT9	M. Yamaizumi
pGBT-hRAD18Δ6BD	human *rad18* (aa 340-395 deletion) with C-terminal myc tag in pGBT9	M. Yamaizumi
pGEX-hUBC9	Full length human *UBC9* in pGEX-6P-1	This study
pGEX-HHR6B	Full length *HHR6B* in pGEX-6P-1	This study
pGEX-hRAD18N	human *rad18* (aa 1-93) in pGEX-6P-2	This study
pGEX-hRAD18C	human *rad18* (aa 94-495) in pGEX-6P-3	This study


Hind III (410)
SnaB I
(4883)
MCS
(1004–1098)
EcoR I
Sma I
BamH I
Sal I
Pst I
P ADH1
2 µ
ori
GAL4
BD
T ADH1
Nae I (1115)
pGBT9
5.5 kb
Aat II
(4109)
Hind III (1319)
Amp r
TRP1
Col E1
ori
Xba I
(1748)
Pvu II
(2118)


Hind III (410)
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MCS
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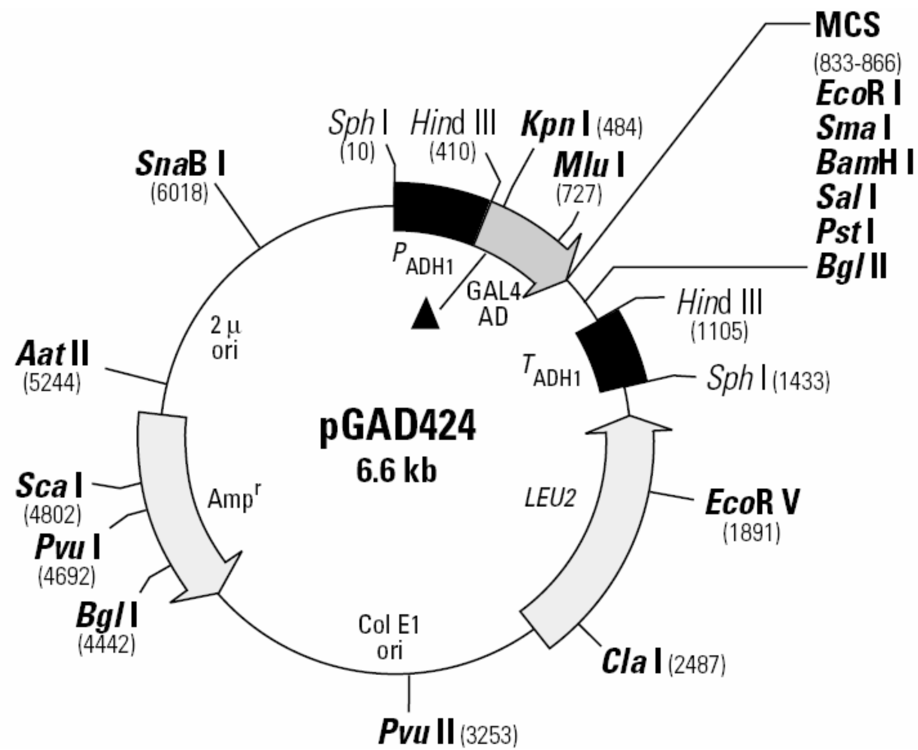
EcoR I
Bgl II
BamH I
Sal I
Pst I
P ADH1
2 µ
ori
GAL4
BD
T ADH1
Nae I (1115)
pGBT9-Bg
5.5 kb
Aat II
(4109)
Hind III (1319)
Amp r
TRP1
Col E1
ori
Xba I
(1748)
Pvu II
(2118)

Fig. 2-1 Map of pGBT9 and pGBT9-Bg vectors. pGBT9-Bg is made by changing *Sma*I site of pGBT9 to *Bgl*II. Both vectors generate a hybrid protein that contains the sequences for the Gal4 DNA-binding domain (aa 1–147). For the construction of a hybrid protein, the gene encoding the protein of interest is ligated into the MCS in the correct orientation and in the correct reading frame such that a fusion protein is generated. The fusion protein is expressed at high levels in yeast host cells from the constitutive *ADH1* promoter and transcription is terminated by the *ADH1* transcription termination signal. The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences that have been added to the AD sequence from a heterologous source (2). pGBT is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae*. It carries the *bla* gene (for ampicillin resistance in *E. coli*) and the *TRP1* selectable marker that allows yeast auxotrophs carrying pGBT9 or pGBT9-Bg to grow on limiting synthetic medium lacking Trp.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EcoR I
Sma I
BamH I
Sal I
Pst I
Bgl II
Sph I
(10)
Hind III
(410)
Kpn I (484)
Mlu I
(727)
SnaB I
(6018)
P ADH1
GAL4
AD
Hind III
(1105)
2 μ
ori
T ADH1
Sph I (1433)
Aat II
(5244)
pGAD424
6.6 kb
Sca I
(4802)
Amp r
LEU2
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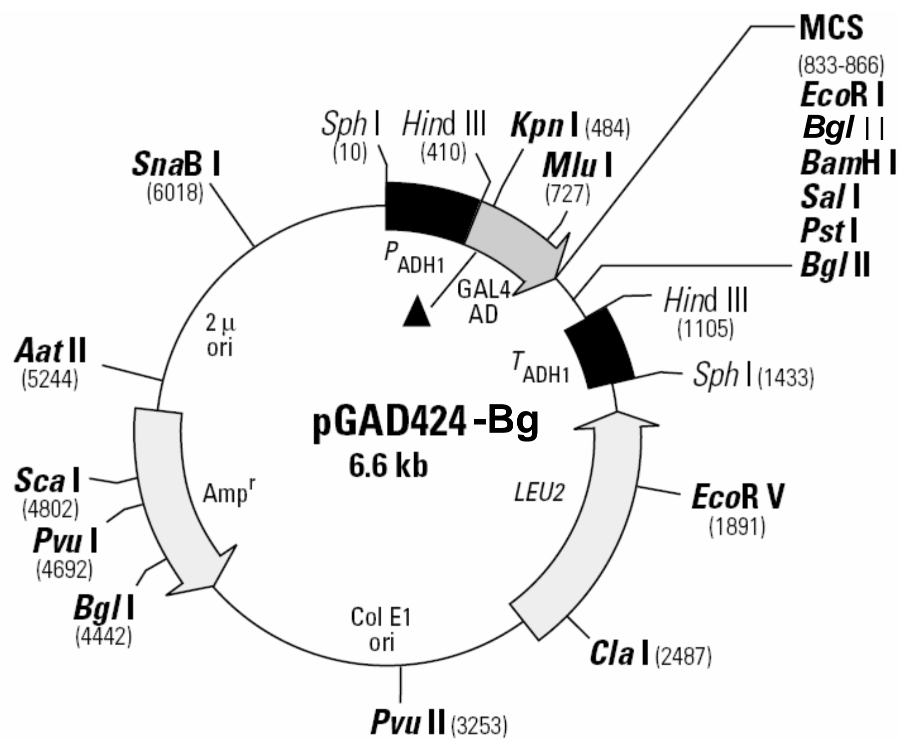
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Hind III
(410)
Kpn I (484)
Mlu I
(727)
SnaB I
(6018)
P ADH1
GAL4
AD
Hind III
(1105)
2 μ
ori
T ADH1
Sph I (1433)
Aat II
(5244)
pGAD424 -Bg
6.6 kb
Sca I
(4802)
Amp r
LEU2
EcoR V
(1891)
Pvu I
(4692)
Bgl I
(4442)
Col E1
ori
Cla I (2487)
Pvu II (3253)

Fig. 2-2 Map of pGAD424 and pGAD424-Bg vectors. pGAD424-Bg is made by changing *Sma*I site of pGAD424 to *Bgl*II. Both vectors generate a hybrid protein that contains the sequences for the Gal4 activation domain (aa 768–881). For the construction of a hybrid protein, the gene encoding the protein of interest is ligated into the MCS in the correct orientation and in the correct reading frame such that a fusion protein is generated. The fusion protein is expressed at high levels in yeast host cells from the constitutive *ADH1* promoter and transcription is terminated by the *ADH1* transcription termination signal. The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences that have been added to the AD sequence from a heterologous source (2). pGAD424 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae*. It carries the *bla* gene (for ampicillin resistance in *E. coli*) and the *LEU2* selectable marker that allows yeast auxotrophs carrying pGAD424 or pGAD424-Bg to grow on limiting synthetic medium lacking Leu.

The yeast expression constructs pEGUh6-HHR6A/B were received from Dr. Z. Wang (Xin *et al.*, 2000), in which human *RAD6A/B* genes were cloned into *Bgl*II and *Hin*dIII sites of pEGUh6 vector. The *Hin*dIII site of pEGUh6-HHR6A/B was converted to *Xho*I by adding a *Xho*I linker to produce pEGUh6-HHR6A/B (Xh). *HHR6A* gene was released from pEGUh6-HHR6A (Xh) by *Nco*I and *Xho*I double digestion and was cloned into the same sites of pACTII to form pACT-HHR6A. *HHR6B* gene was released from pEGUh6-HHR6B (Xh) by *Bgl*II and *Xho*I double digestion and was cloned into *Bgl*II and *Sal*I sites of pGAD424-Bg to form pGAD-HHR6B.

A 1.5 Kb *Bam*HI-*Sal*I fragment of human *RAD18* gene was cloned into the same sites of pGBT9-Bg and pGAD424-Bg to form pGBT-hRAD18 and pGAD-hRAD18 respectively. Plasmids pGBT-hRAD18N (aa 1-93) and pGAD-hRAD18N (aa 1-93) were made by digestion of pGBT-hRAD18 and pGAD-hRAD18 with *Pst*I followed by self ligation. To obtain the plasmid pGBT-hRAD18C (aa 94-495), the 1.2 Kb *Pst*I fragment from pGBT-hRAD18 was cloned in the same site of pGBT9-Bg.

Yeast two-hybrid constructs pGBT-myc hRAD18, pGBT-hRAD18C28F, pGBT-hRAD18C207F, and pGBT-hRAD18Δ6BD are from Dr. M. Yamaizumi.

2.2.10.2 Plasmids for protein expression

The pGEX-6P vectors were used to express proteins of interest in *E. coli*. pGEX-6P-1, pGEX-6P-2, and pGEX-6P-3 each encode the recognition sequence for site-specific cleavage by PreScission Protease between Glutathione S-transferase (GST)

domain and the multiple cloning sites (MCS) (Fig. 2-3). Inserting a gene of gene fragment into MCS allows the expression of a fusion protein with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. Expression is under the control of the *tac* promoter, which is induced by the lactose analog IPTG. All pGEX-6P vectors are also engineered with an internal *lac*I^q gene. The *lac*I^q gene product is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of the inserted gene or gene fragment.

The human *UBC9* gene fragment was released from pGAD-hUBC9 by *Eco*RI and *Sal*I double cleavage and cloned into the same sites of pGEX-6P-1 to form pGEX-hUBC9.

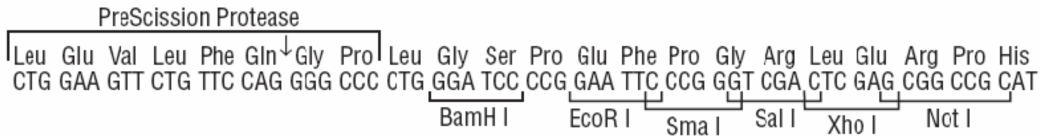
The *Bgl*II fragment from pGAD-hRAD18N (aa 1-93) was cloned in the *Bam*HI site of pGEX-6P-2 to produce pGEX-hRAD18N (aa 1-93). The *Sal*I fragment from pGBT-hRAD18C (aa 94-495) was cloned in the same site of pGEX-6P-3 to produce pGEX-hRAD18C (aa 94-495).

HHR6B ORF fragment was isolated from pEGUh6-HHR6B (Xh) by *Bgl*II and *Xho*I double cleavage and was cloned into the *Bam*HI and *Xho*I sites of pGEX-6P-1 to produce pGEX-HHR6B.

2.2.11 Protein expression and purification

2.2.11.1 Soluble expression of GST fusion protein

pGEX-6P-1 (27-4597-01)



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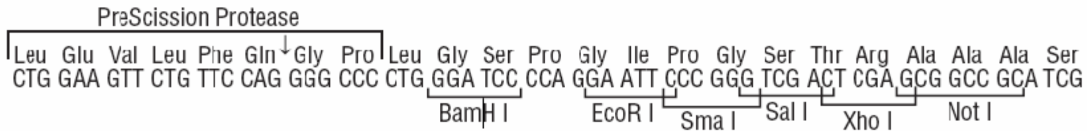

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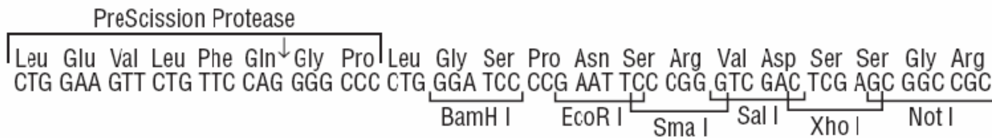

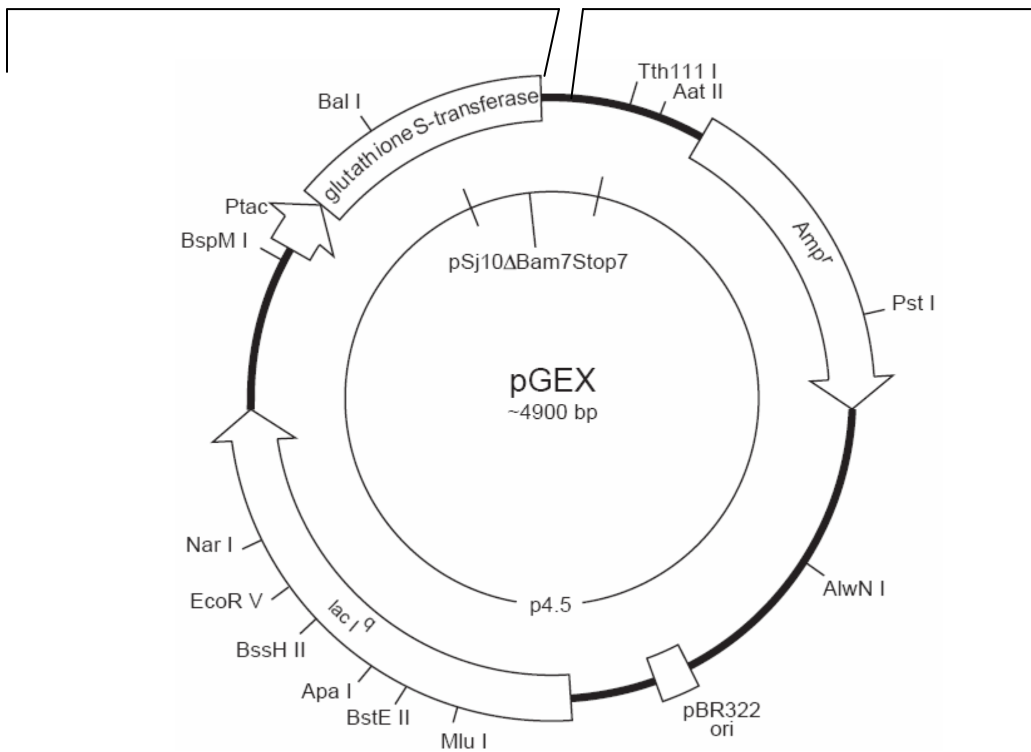

Fig. 2-3 Map of the pGEX vectors showing the reading frames and main features. The vectors have an expanded MCS that contains six restriction sites. The pGEX-6P series provides all three translational reading frames linked between the GST coding region and the MCS.

The pGEX recombinant plasmids pGEX-HHR6B, pGEX-hUBC9, and pGEX-hRAD18C (aa 94-495) were transformed into *E. coli* strain BL21-RIL. A single colony was inoculated into 50 ml LB broth with 100 µg/ml ampicillin and shaked at 37°C overnight. 10 ml overnight cell cultures were used to inoculate 1 liter LB broth with 100 µg/ml ampicillin. The cells were grown at 37°C to an OD_{600} of 0.6-1.0. The recombinant plasmid was induced to express fusion protein by adding IPTG to a final concentration of 0.2 mM to 1.0 mM. Cells were incubated for additional 2-3 hours and were harvested by centrifugation at 5,000 x *g* for 5 minutes at 4°C.

2.2.11.2 GST fusion protein purification

The cell pellet harvested in **2.2.11.1** was resuspended in 30-50 ml 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3). Cells were disrupted by French Press. The fusion proteins accumulated within the cell cytoplasm were released into cell supernatant. The supernatant was recovered from the cell lysis by centrifuging at 15,000 x *g* for 30 minutes at 4°C and the pellet was discarded. After filtered by 0.45 µm filter, the supernatant was loaded onto GSTrap FF 5 ml column (Amersham Biosciences) pre-washed by wash buffer (1X PBS). Fusion proteins which remained on the column during loading were eluted by elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) and collected.

2.2.11.3 GST fusion protein cleavage and purification of released protein

The purified fusion proteins were dialyzed against cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH7.0) to remove free reduced glutathione. Then the protein sample was cleaved by PreScission Protease (Amersham Biosciences) at 4°C for 16 hours. Once digestion is completed, the sample was applied to GSTrap FF 5 ml column. The GST moiety of the fusion protein and PreScission Protease remained bound to the column and the protein of interest was in the flow-through.

2.2.11.4 Expression and isolation of inclusion body form GST-hRad18N

The plasmid pGEX-hRAD18N was transformed into *E. coli* strain BL21-RIL for expression of GST-hRad18N in its inclusion body form. The transformed BL21 cells were grown in 600 ml of LB broth with 100 μg/ml ampicillin at 37°C to OD_{600} of 0.6. IPTG was added to a final concentration of 0.2 mM to induce the protein expression. Following additional incubation for 2 hours, cells were collected by centrifugation, resuspended in 20 ml 1 X PBS. The cells were lysed using a French Press. The inclusion bodies was harvested from the pellet by centrifugation, resuspended in 50 ml of 50 mM Tris-HCl, pH 8.0, 20.0 mM EDTA and sonicated for three minutes on ice. Inclusion bodies were then harvested, washed 3 times with the same buffer and stored at -20°C to use.

2.2.11.5 Denaturation, refolding and recovery of GST-hRad18N

A process of solubilization and renaturation modified from Buchner and Rudolph

(Buchner and Rudolph, 1991) was used for the recovery of soluble GST-hRad18N. Inclusion bodies were dissolved in solubilization buffer (6.0 M guanidine, 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 300 mM DTT) and centrifuged at 14,000 g for 10 minutes to remove the particles. The solubilized inclusion bodies were diluted 100-fold in 0.1 M Tris-HCl buffer, pH 8.0, 0.5 M L-arginine, 8 mM oxidized glutathione (GSSG), 2 mM EDTA, for a final DTT:GSSG ratio of 3:4. The solution was incubated at 10°C for 48 hours. The sample was concentrated 10 times by a 10 kDa cut off ultrafiltration filter (Amicon) and then dialyzed against 10 volumes of 20 mM sodium phosphate buffer, pH 6.8, 50 mM NaCl with three changes.

2.2.12 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Proteins were separated and visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli's method as described by Sambrook *et al.* (1989). Gels were prepared with a 12% acrylamide separating gel bed topped with a 4% stacking gel. For one mini-gel (6 cm X 8 cm), the separating and stacking gel mixtures were prepared as follows: 5 ml of separating gel with 12% acrylamide (29:1 acrylamide:bis-acrylamide), 0.1% (w/v) SDS and 373 mM Tris-HCl buffer, pH 8.8; and 2 ml of 4% stacking gel with 4% acrylamide (29:1), and 0.1% (w/v) SDS and 125 mM Tris-HCl buffer, pH 6.8. Polymerization was initiated with 0.05% (w/v) AP and 0.05% (v/v) TEMED just before use. The running gel was poured between plates separated with 1 mm spacers, anchored and sealed in a Bio-Rad mini gel caster, and

topped with water for an even level gel surface. After polymerization was complete, water was drained out and stacking gel was poured over the running gel. The comb was placed and the gel was allowed to polymerize.

Gels were clamped into the electrophoresis apparatus. Both the top and bottom buffer chambers were filled with 1 X Tris-glycine buffer [25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS] and the combs were removed. Samples were mixed 1:1 with standard 2X gel-loading buffer [200 mM DTT, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 100 mM Tris-HCl, pH 6.8]. Samples were boiled for 5 minutes prior to loading. Gels were run at constant voltage, 180V, for about 1 hour, using bromophenol blue as a marker. The gel was stained for 30 minutes in Coomassie stain, 0.25% (w/v) Coomassie brilliant blue R250 dissolved in 40% methanol (v/v), 10% (v/v) acetic acid in water. Gels were photographed wet.

2.2.13 Protein interaction analysis by pull-down experiment

2.2.13.1 MicroSpin GST column preparation

The bottom of MicroSpin GST column (Amersham Biosciences) was broken and the column was inserted into a 1.5 mL eppendorf tube. The tube was centrifuged at 3,000 rpm for 30 seconds and the flow-through from the column was discarded. The column was washed with 500 µl 1 X PBS and the flow-through was discarded.

2.2.13.2 *In vitro* binding assay

Two MicroSpin GST columns were prepared as above. 200 µl of GST (as control) and GST fusion protein were added to the columns, respectively. After incubation at 4°C for 1 hour with slow shaking, the columns were spun and the flow-throughs were collected (referred as flow-through1). The columns were then PBS washed and the wash buffer was collected after centrifugation (referred as flow-through2). Two hundred µl of protein without GST fusion were added to the columns. After incubation at 4°C for 1 hour with slow shaking, the columns were centrifuged and the flow-throughs were collected (referred as flow-through3). The columns were then PBS washed two times and 100 µl of 10 mM reduced glutathione were added to elute the proteins bound to the column. After incubation at 4°C for 10 minutes, the columns were centrifuged and the flow-throughs were collected (referred as eluate). The collected samples were SDS-PAGE analyzed.

CHAPTER THREE

RESULTS

3.1 Mapping the interaction domain in the yeast Rad18-Rad6 complex

Gal4 contains a DNA-binding domain ($Gal4_{BD}$) within amino acids 1-147 and an activation domain ($Gal4_{AD}$) within amino acids 768-881. The coding regions for these two domains are carried on plasmids pGBT9 and pGAD424, respectively (Chien *et al.*, 1991; Fields and Song, 1989). Strain Y190 used for the two-hybrid assay carries a *GAL1-lacZ* fusion gene which contains an UAS (upstream activation sequence) in the promoter region of *GAL1*. The Gal4 DNA-binding domain is able to bind to the UAS. Interaction between a protein or a peptide fused to $Gal4_{BD}$ and a second protein fused to the $Gal4_{AD}$ directs $Gal4_{AD}$ to the UAS site, resulting in β-galactosidase expression, which can be detected and measured by a β-gal filter assay. Another strain used is PJ69-4A in which a *HIS3* gene under control of the *GAL1* promoter was introduced into the genome (James *et al.*, 1996). Interaction between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$ will drive the expression of *HIS3* gene, resulting in the growth of this strain on SC minus His media. This *GAL1-HIS3* reporter worked well. 0-2 mM 3-aminotriazole (3-AT) is sufficient to eliminate false positives due to leakiness on SC minus His media. These low level of 3-AT have no effect on the growth of true positives, making this a very sensitive reporter.

The *RAD6* and *RAD18* genes of *S. cerevisiae* are the two most important members

in PRR. Rad6 mediates its role in DNA repair through forming a complex with Rad18 (Bailly *et al.*, 1994). The Rad18 has several putative domains (Fig. 3-1) and is a RING finger E3. Bailly *et al.* reported that 40 amino acids from 371 to 410 of Rad18 interacted efficiently with Rad6 (Bailly *et al.*, 1997b). In this study, I used yeast two-hybrid assay to test the ability of the 40 amino-acid peptide interaction with Rad6. A series of $Gas4_{AD}$-Rad18 mutant constructs and $Gal4_{BD}$-Rad18-40 were made (Fig. 3-1). To carry out the yeast two-hybrid assay, these constructs were co-transformed with $Gal4_{BD}$ /$Gal4_{AD}$-Rad6 into yeast strain Y190 or PJ69-4A. As shown in Fig. 3-2, Rad18 interacts with Rad6 *in vivo*. The C-terminal deletion of Rad18 made it unable to interact with Rad6, which indicated the interacting domain is at the C-terminus. A 40 aa fragment from residues 371 to 410 is sufficient for the interaction with Rad6 (Fig. 3-3). This result is consistent with Bailly's report (Bailly *et al.*, 1997b). However, a key Cys mutation (Cys to Ser) in RING finger domain of Rad18 completely abolished its interaction with Rad6 (Fig. 3-2). One possibility which caused the lack of interaction is that Rad18C28S is unstable in yeast cells and degraded rapidly.

3.2 Mapping the interaction domain in the human Rad18-Rad6 complex

In budding yeast, Rad18 binds to Rad6 through its **R**ad**6**-**b**inding **d**omain (R6BD) (Bailly *et al.*, 1997b). This domain is highly conserved among various species and this putative R6BD motif in hRad18 is from aa 340 to 395. In previous study, plasmid containing hRAD18 gene but lacking sequence encoding R6BD domain was used to

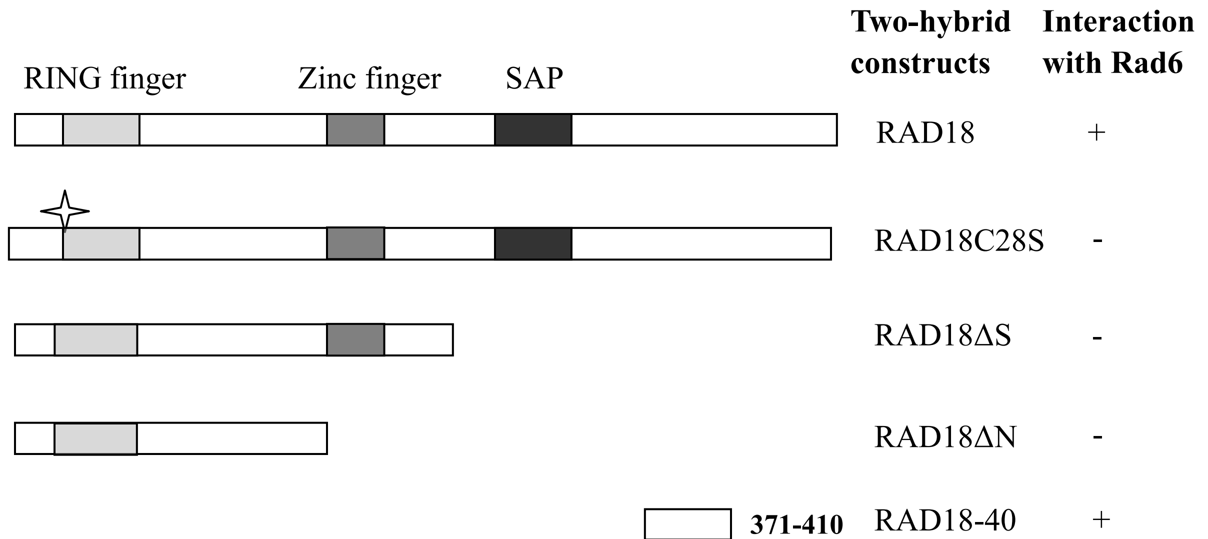

Fig. 3-1 Rad18 fragments cloned into yeast two-hybrid vectors and their interactions with Rad6 in yeast two-hybrid assays. RAD18 gene was truncated to produce different fragments which were cloned into yeast two-hybrid vectors pGBT9 or pGAD424. The putative domains of Rad18, RING finger, Zinc finger and SAP, are shown in each fragment. The ✧ symbol stands for a point mutation Cys28 mutated to Ser. The number denotes the amino acids of the fragment in Rad18 protein. "+" indicates an interaction between Rad18 or its mutants and Rad6, while "-" indicates no interaction.

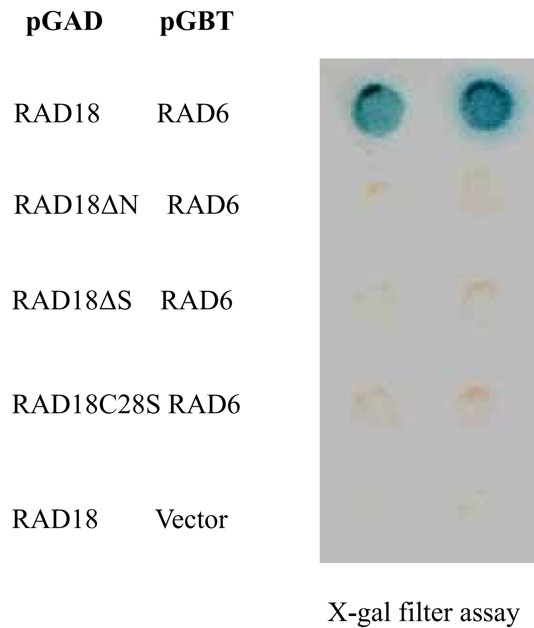

Fig. 3-2 Yeast two-hybrid assay of interactions between Rad18 or its mutants and Rad6. Yeast strain Y190 was co-transformed with pGBT-RAD6 and pGAD-RAD18 derivatives, and the co-transformants were used for β-gal assay. Y190 co-transformed with pGBT9 and pGAD-RAD18 was used as a negative control. Spots with color turned blue indicate interaction; spots without color change indicate no interaction.

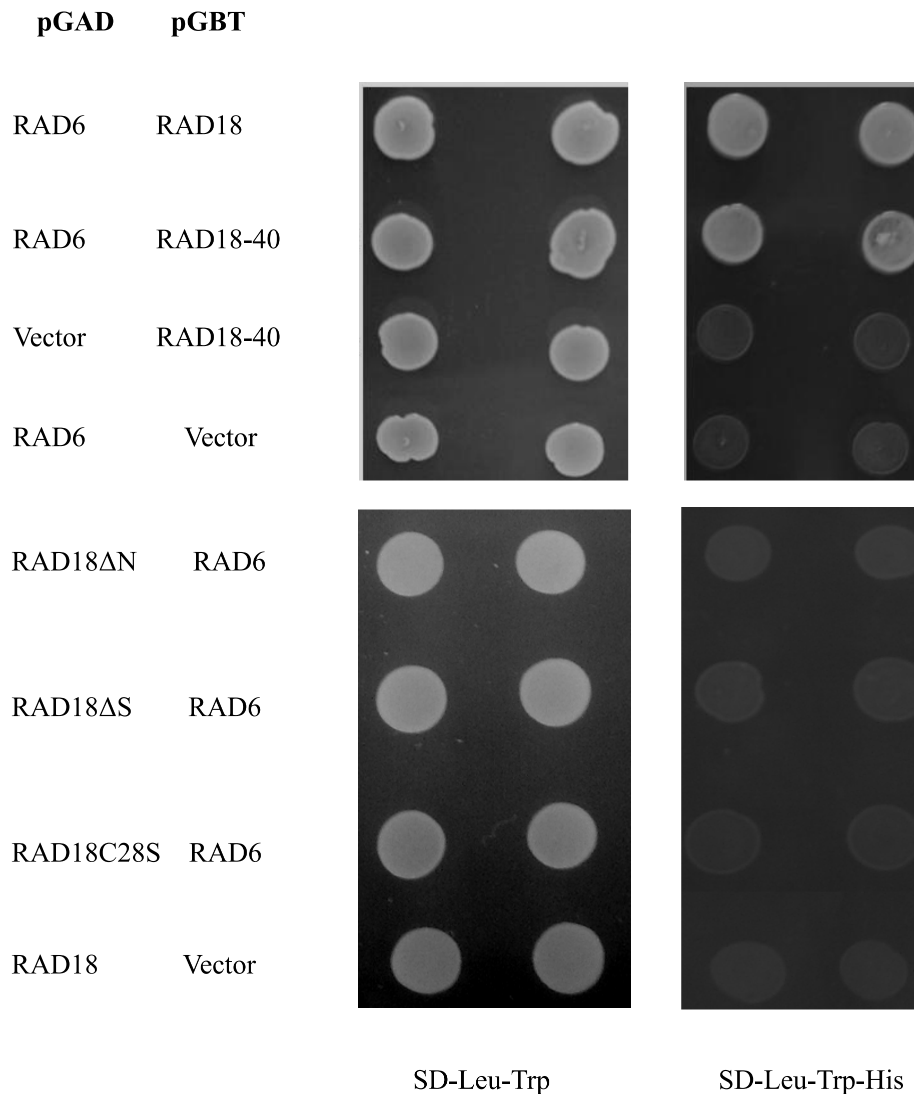

Fig. 3-3 Yeast two-hybrid assay of interactions between Rad18 or its mutants and Rad6. Yeast strain PJ69-4A was transformed with different combinations of pGBT and pGAD constructs. All transformants grow on SD-Leu-Trp plates for selection. The same transformants were spotted on SD-Leu-Trp-His plates to test the activation of the *HIS3* gene. The transformants which grew on SD-Leu-Trp-His plates indicated interactions between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$. Negative controls were shown.

transfect COS-7 cells together with plasmids containing HHR6A/B gene. Co-immunoprecipitation experiments showed this mutated hRad18 localized in the nuclei like WT Rad18, but did not interact with HHR6A/B (Watanabe *et al.*, 2004). However, there is no direct evidence to show R6BD is the domain of hRad18 interacting with HHR6A/B.

In this study, yeast two hybrid assays were used to determine the domain required for hRad18 and HHR6A/B interaction. To achieve the goal, a series of hRAD18 gene fragments or mutations were cloned into yeast two hybrid vectors (Fig. 3-4).

The interaction between hRad18 and HHR6A/B was confirmed by the yeast two-hybrid assays (Fig. 3-5). Further studies indicated that HHR6A/B did not interact with the N-terminal fragment of hRad18, but interacted with the C-terminal fragment which contained R6BD (Fig. 3-6). The R6BD deletion (Δ6BD) totally abolished the interaction of these two proteins, but Zinc finger domain mutation, hRad18C207F, did not (Fig. 3-6). The results supported that the R6BD of hRad18 is required for interacting with HHR6A/B. RING finger domain mutation (hRad18C28F) also eliminated the interaction between hRad18 and HHR6A/B, which is controversial to the results that HHR6A/B did not interact with the N-terminal fragment of hRad18. The reason of the opposite results may be that the key cysteine mutation in the RING finger domain made the motif unable to bind zinc ions; thus this altered protein cannot fold correctly, and is subject to degradation by proteasome.

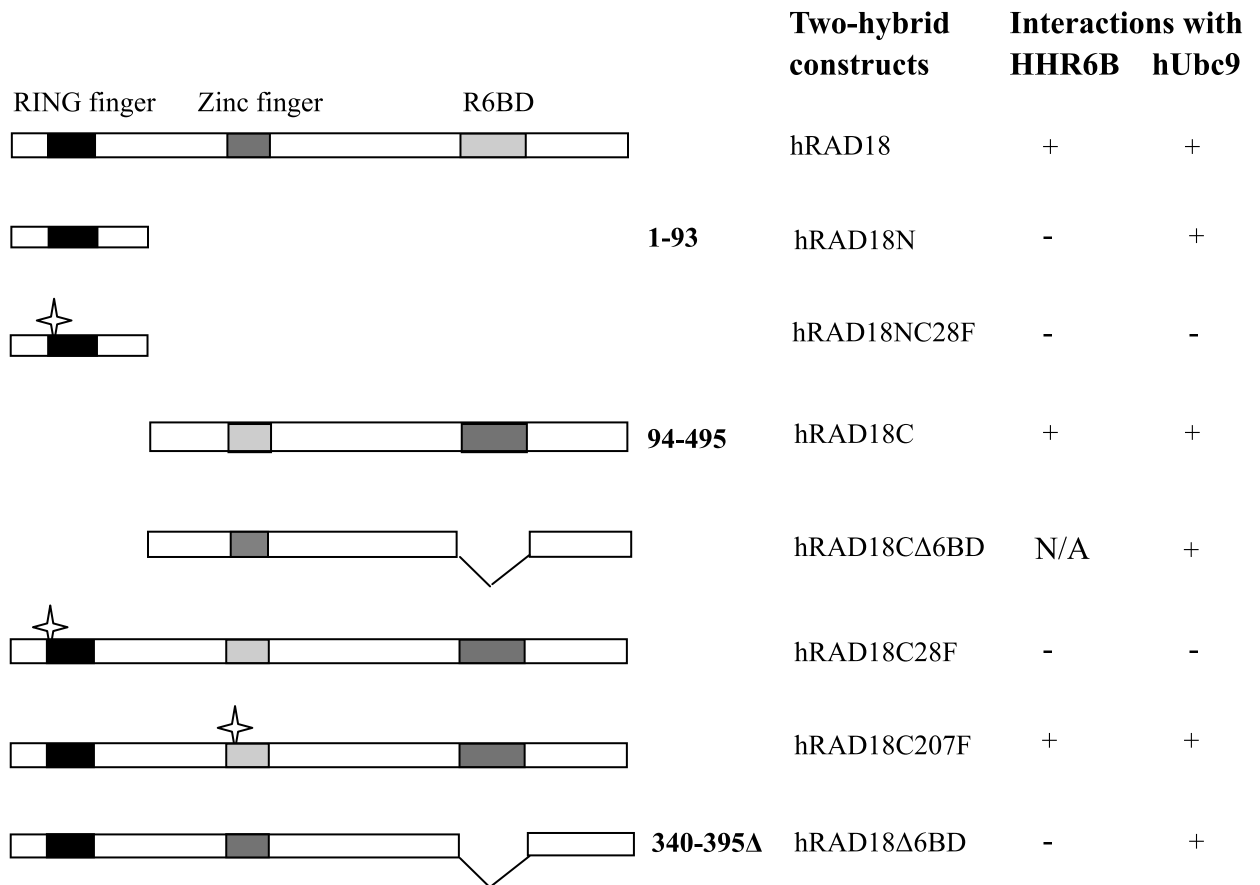

Fig. 3-4 Human Rad18 fragments and mutants cloned into yeast two-hybrid vectors. hRAD18N is the N-terminal fragment of hRAD18 containing RING finger domain. hRAD18NC28F is the hRAD18N with RING finger mutation. hRAD18C is the C-terminal fragment containing Zinc finger and R6BD. hRAD18CΔ6BD is the hRAD18C with R6BD domain deleted. hRAD18C28F and hRAD18C207F are the mutations which have a key cysteine in RING finger or Zinc finger domain mutated to phenylalanine. hRAD18Δ6BD is the full length hRAD18 gene without sequences encoding R6BD domain. The number denotes the amino acids of the fragment in hRad18 protein. "+" indicates an interaction between hRad18 or its mutants and HHR6B or hUbc9, while "-" indicates no interaction.

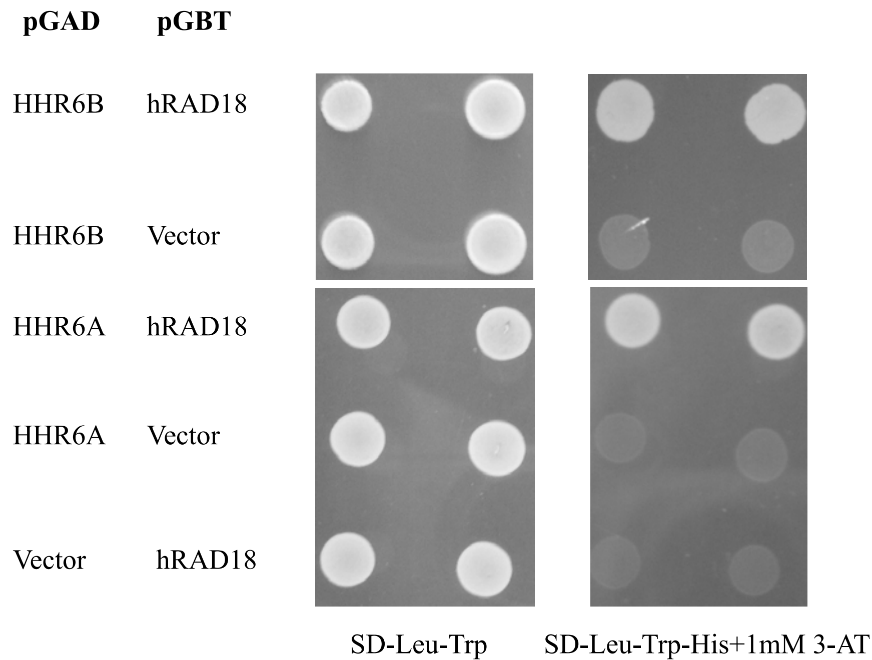

Fig. 3-5 Yeast two-hybrid assay of interactions between human Rad18 and HHR6A/B.

Yeast strain PJ69-4A was transformed with different combinations of pGBT and pGAD constructs. All transformants grow on SD-Leu-Trp plates for selection. The same transformants were spotted on SD-Leu-Trp-His plates containing 1 mM 3-AT to test the activation of the *HIS3* gene. The transformants which grew on SD-Leu-Trp-His plates indicated interactions between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$. Negative controls were shown.

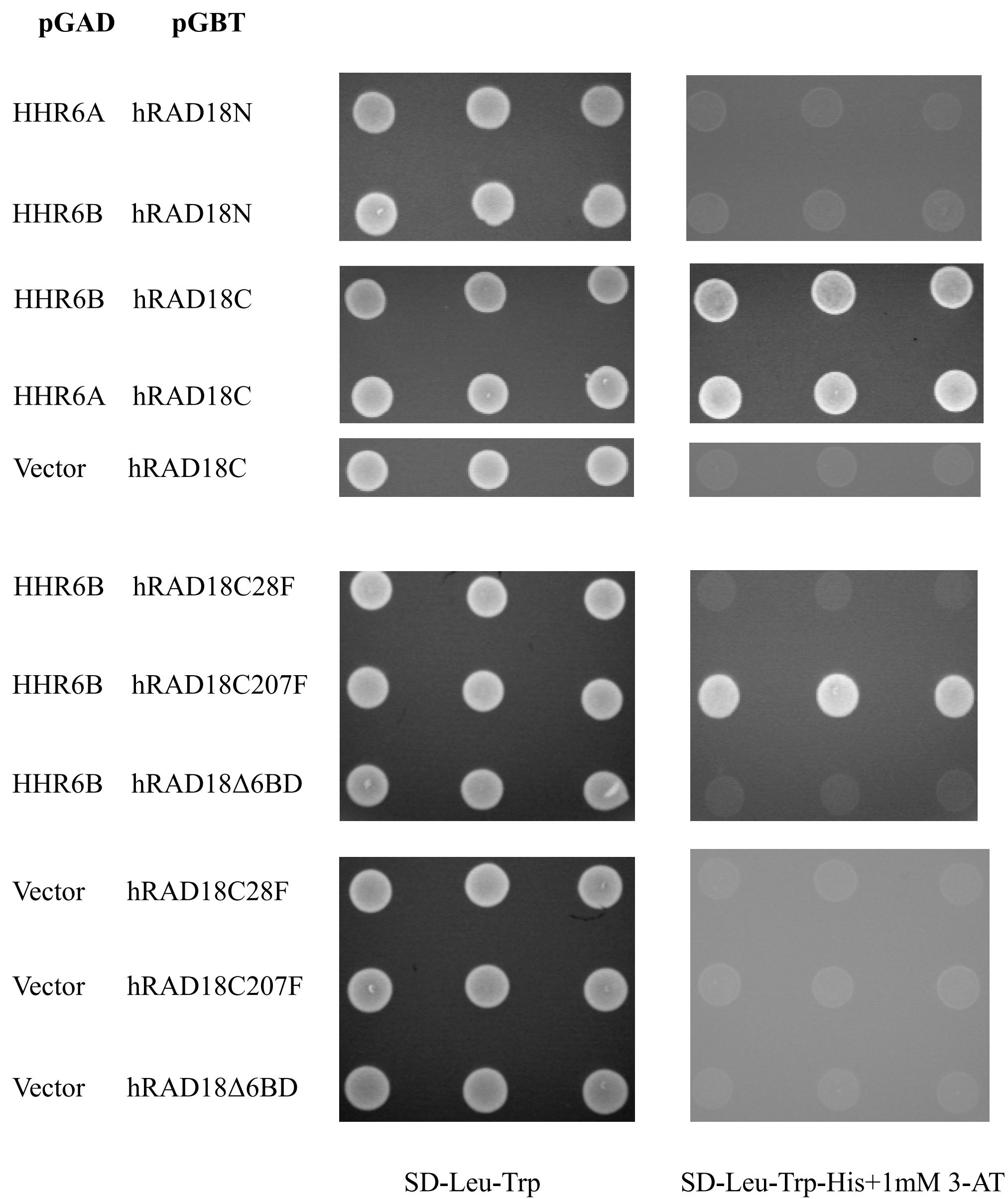

Fig. 3-6 Yeast two-hybrid assay of interactions between human Rad18 mutants and HHR6A/B. Yeast strain PJ69-4A was transformed with different combinations of pGBT and pGAD constructs. All transformants grew on SD-Leu-Trp plates for selection. The same transformants were spotted on SD-Leu-Trp-His plates containing 1 mM 3-AT to test the activation of the *HIS3* gene. The transformants which grew on SD-Leu-Trp-His plates indicated interactions between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$. Negative controls were shown.

3.3 Mapping interaction domain of human Rad18 interacting with human Ubc9

The interaction between yeast Rad18 and Ubc9 was first shown in Hoege's paper (Hoege *et al.*, 2002). This interaction was confirmed in my yeast two-hybrid analysis (Fig. 3-7). Due to the high aa sequence identity between yeast Ubc9 and human Ubc9, hUbc9 can interact with yeast Rad18, too (Fig. 3-8).

The interaction between hRad18 and hUbc9 was found in my yeast two hybrid assay (Fig. 3-7). When hUbc9 was fused to $Gal4_{BD}$ domain, the interaction between hRad18 and hUbc9 was not detected (data not shown). So I chose $Gal4_{AD}$-hUBC9 to study the domain(s) of hRad18 interacting with hUbc9. Human Ubc9 interacts with both the N-terminal and the C-terminal fragment of hRad18 (Fig. 3-8), which suggests that hRad18 has two domains interacting with hUbc9.

Human Ubc9 can also interact with the N-terminal fragment of yeast Rad18, Rad18ΔS and Rad18ΔN (Fig. 3-8). The N-terminal fragments, yeast Rad18ΔN (see Fig. 3-1) and human Rad18N, contain only a RING finger domain. A key cysteine mutation in the RING finger domain, yeast Rad18C28S (Fig. 3-8) and human Rad18C28F (Fig. 3-9), abolished the two proteins' interaction. This may be caused by the instability and degradation of the mutated proteins in vivo. Further studies need to find out whether RING finger domain is one of the two domains of hRad18 interacting with hUbc9. The Zinc finger domain mutation (hRad18C207F) and R6BD deletion (hRad18Δ6BD) did not abolish the interaction between these two proteins (Fig. 3-9A). Because there is a domain

interacting with hUbc9 at the N-terminus of hRad18, we do not know if the Zinc finger or the R6BD domain is involved in interaction with hUbc9 from results that hRAD18Δ6BD and hRAD18C207F interacted with hUbc9. To test whether Δ6BD is involved in hRad18 and hUbc9 interaction, we made the construct hRAD18CΔ6BD which does not contain the N-terminal interacting domain of hRad18 and has the Δ6BD domain deletion. Fig. 3-9B showed hRAD18CΔ6BD can still interact with hUbc9, which indicated that R6BD is not involved in the interaction of hRad18 and hUbc9. Further studies need to be performed to find out the exact domain at the C-terminus of hRad18 interacting with hUbc9.

3.4 Protein expression and purification

The interactions between hRad18N/hRad18C and hUbc9 were shown in yeast two-hybrid assays, and hRad18C was also shown to interact with HHR6B. To confirm their interactions, these proteins or peptides were purified in GST fusion form or in free form to do *in vitro* binding experiments. GST fusions were used as "bait" proteins to pull down free proteins.

HHR6B and hUbc9 fusion proteins were expressed in soluble form, and purified by affinity chromatography. HHR6B and hUbc9 were further purified after cleavage of GST from the fusion proteins. The C-terminal fragment of human Rad18 fused to GST (GST-hRad18C) was expressed in soluble form and purified. GST-hRad18N was expressed as inclusion bodies. It was recovered by denaturation and renaturation.

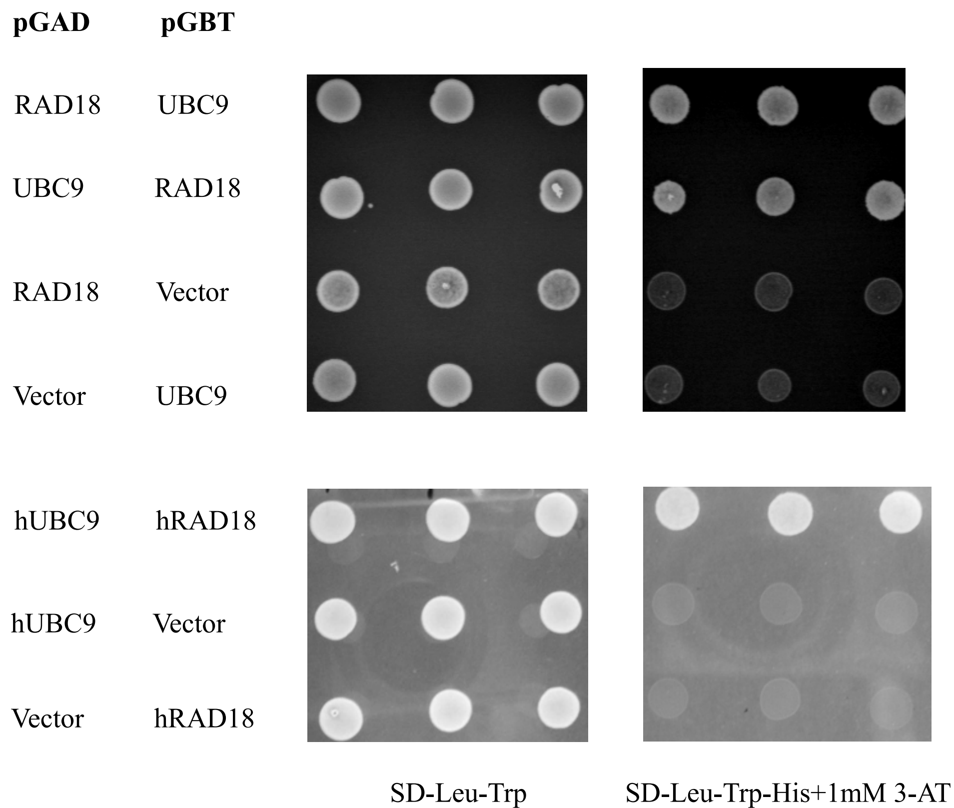

Fig. 3-7 Yeast two-hybrid assay of interactions between human/yeast Rad18 and Ubc9. Yeast strain PJ69-4A was transformed with different combinations of pGBT and pGAD constructs. All transformants grow on SD-Leu-Trp plates for selection. The same transformants were spotted on SD-Leu-Trp-His plates containing 1 mM 3-AT to test the activation of the *HIS3* gene. The transformants which grew on SD-Leu-Trp-His plates indicated interactions between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$. Negative controls were shown.

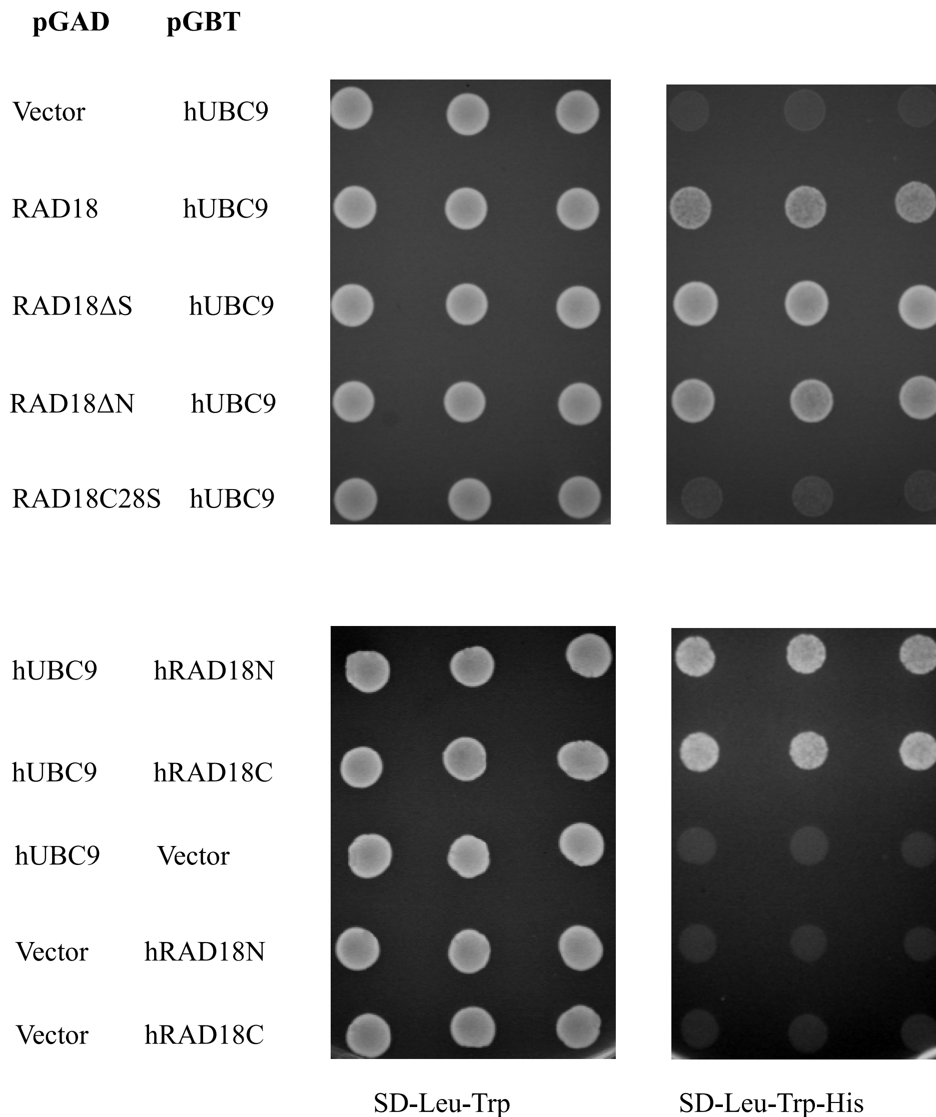

Fig. 3-8 Yeast two-hybrid assay of interactions between human Ubc9 and human/yeast Rad18 mutants. Yeast strain PJ69-4A was transformed with different combinations of pGBT and pGAD constructs. All transformants grow on SD-Leu-Trp plates for selection. The same transformants were spotted on SD-Leu-Trp-His plates containing 1 mM 3-AT to test the activation of the *HIS3* gene. The transformants which grew on SD-Leu-Trp-His plates indicated interactions between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$. Negative controls were shown.

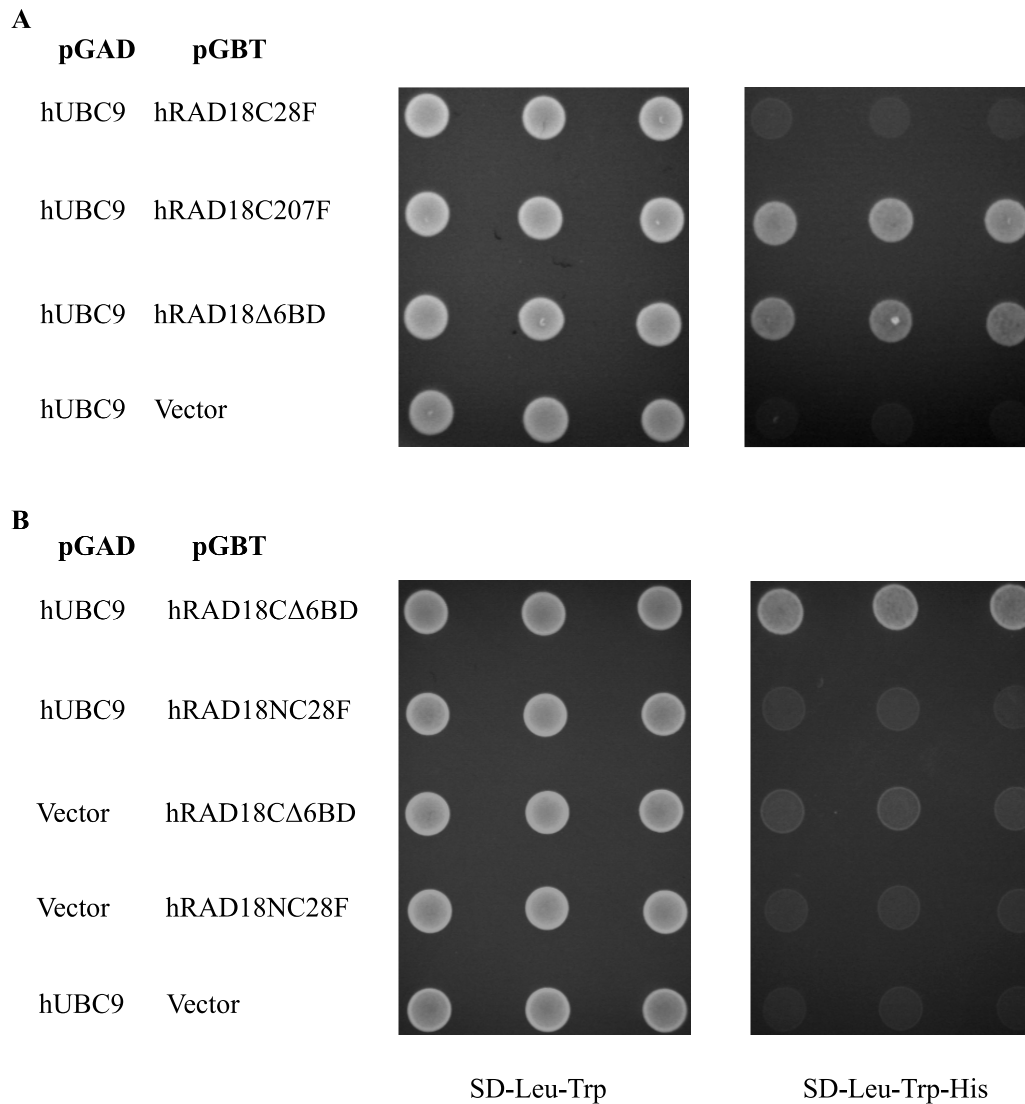

Fig. 3-9 Yeast two-hybrid assay of interactions between human Ubc9 and human Rad18 mutants. Yeast strain PJ69-4A was transformed with different combinations of pGBT and pGAD constructs. All transformants grew on SD-Leu-Trp plates for selection. The same transformants were spotted on SD-Leu-Trp-His plates to test the activation of the *HIS3* gene. The transformants which grew on SD-Leu-Trp-His plates indicated interactions between proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$. Negative controls were shown.

3.4.1 Expression and purification of HHR6B and hUbc9

HHR6B and hUbc9 proteins were expressed in soluble form and yielded large amount of GST fusion proteins. The fusion proteins were purified by affinity chromatography (lane 1 of Fig. 3-10 and lane 4 of Fig. 3-11). Due to autocleavage, some GST proteins were in the purified solutions. After dialysis against cleavage buffer, fusion proteins were cleaved by PreScission Protease. As shown in lane 2 of Fig. 3-10 and lane 3 of Fig. 3-11, the fusion proteins were completely cleaved to release two smaller proteins: GST and HHR6B or hUbc9. The cleaved samples were loaded onto Glutathione Sepharose column again, and the proteins of HHR6B and hUbc9 did not bind to the column and were collected in the flow-through (lane 3 of Fig. 3-10 and lane 2 of Fig. 3-11) while GST and PreScission Protease remained on the column.

3.4.2 Expression and purification of GST-hRad18C

The construct of pGEX-hRAD18C was transformed into *E coli* strain BL21-RIL. The induction was carried out at RT with a low IPTG concentration (0.1~0.2 mM) in case the fusion protein formed inclusion body. A band of 100 kDa was accumulated in induced cells transformed with pGEX-hRAD18C compared to non-induced cells (lanes 3 and 4 of Fig. 3-12). Soluble form of GST-hRad18C was purified by affinity chromatography (lane 2 of Fig. 3-12). As shown in Fig. 3-12 (lane 2), the collected eluate contained many small bands. These bands are probably the partially degraded GST-hRad18C, which suggests this protein is easily subject to degradation.

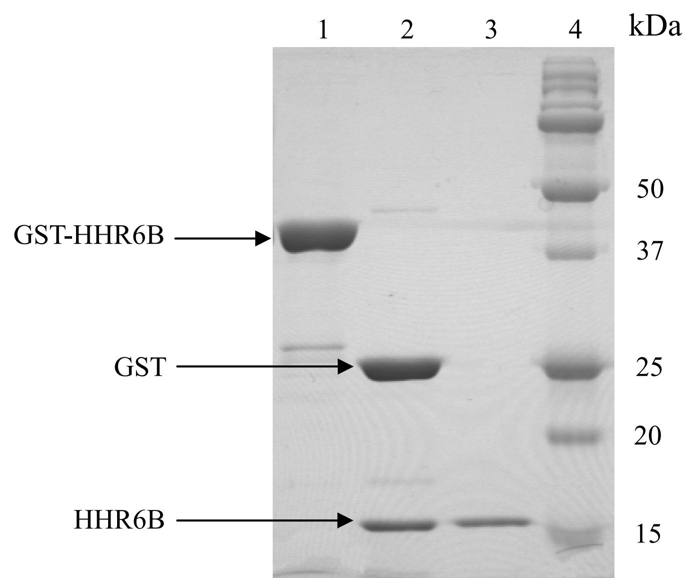

Fig. 3-10 SDS-PAGE analysis of purified HHR6B protein. Lane 1: purified GST-HHR6B. Lane 2: GST-HHR6B cleaved by PreScission Protease. Lane 3: purified HHR6B. Lane 4: protein molecular weight markers. The samples were run on a 12% polyacrylamide gel and the gel was then Coomassie blue stained.

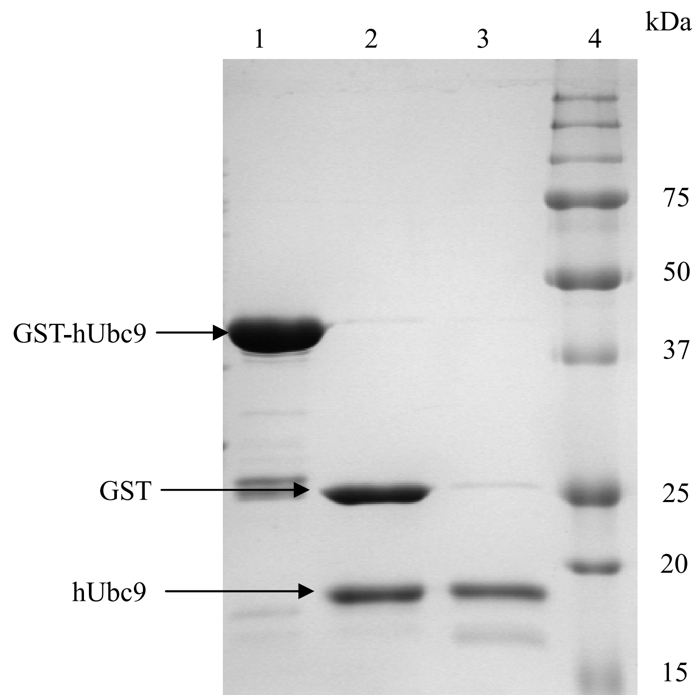

Fig. 3-11 SDS-PAGE analysis of purified human Ubc9 protein. Lane 1: purified GST-hUbc9. Lane 2: GST-hUbc9 cleaved by PreScission Protease. Lane 3: purified hUbc9. Lane 4: protein molecular weight markers. The samples were run on a 12% polyacrylamide gel and the gel was then Coomassie blue stained.

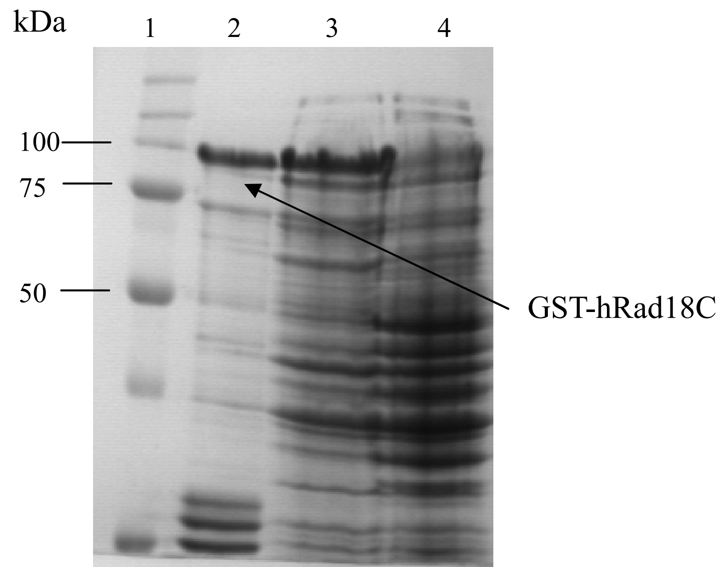

Fig. 3-12 SDS-PAGE analysis of GST-hRad18C. Lane 1: protein molecular weight markers. Lane 2: Purified GST-hRad18C. Lane 3: total protein from induced BL21/pGEX-hRAD18C cells. Lane 4: total protein from non-induced BL21/pGEX-hRAD18C cells. The samples were run on a 12% polyacrylamide gel and the gel was then Coomassie blue stained.

3.4.3 Insoluble expression and purification of GST-hRad18N

The expression vector pGEX-hRad18N was transformed into BL21-RIL cells. A band of about 37 kDa, equal to the size of GST-hRad18N, accumulated in induced cells (lane 2 of Fig. 3-13A) compared to non-induced cells (lane 1 of Fig. 3-13A). Further analysis indicated that the fusion protein formed inclusion body. The inclusion bodies were harvested from cell extracts. A process of solubilization and renaturation was used to recover GST-hRad18N. The protein was finally refolded in refolding buffer and was subsequently concentrated. As shown in Fig. 3-13B, the concentrated solution contained a strong band of 37 kDa. This band was detected by GST antibody (data not shown), and it could bind to GST column when it was used in pull-down experiment (see section **3.5**).

3.5 Pull-down experiments

GST-hRad18N and GST-hRad18C were used to pull down hUbc9 in the in vitro binding experiments. The fusion proteins were first loaded onto MicroSpin GST column. After incubation, the column was centrifuged and flow-through was collected. Lane 1 and 5 of Fig. 3-14 showed no or trace GST-hRad18N and GST-hRad18C proteins in the flow-through, which indicated that the fusion proteins were bound to the columns. The column was then PBS washed and flow-through was collected. Lane 2 and 6 of Fig. 3-14 showed no free fusion proteins were remaining in the column. Human Ubc9 protein was added into the column, and incubated. The column was centrifuged and flow-through was collected. After PBS wash, proteins remaining on the column were eluted by reduced

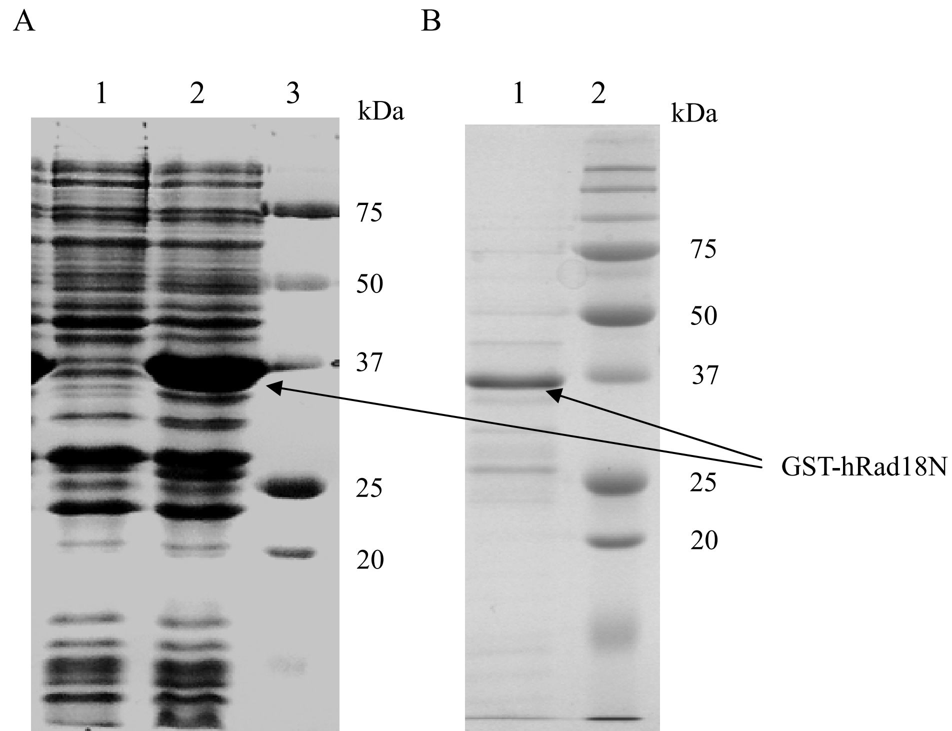

Fig. 3-13 SDS-PAGE analyses of GST-hRad18N inclusion bodies. (A) Lane 1: total protein from non-induced BL21/pGEX-hRAD18N Lane 2: total protein from induced BL21/pGEX-hRAD18N. Lane 3: protein molecular weight markers. (B) Lane 1: GST-hRad18N protein recovered from inclusion body. Lane 2: protein molecular weight markers. The samples were run on a 12% polyacrylamide gel and the gel was then Coomassie blue stained.

glutathione. The eluate was collected. The hUbc9 protein was in the flow-through (lane 3 and 7 of Fig. 3-14). The fusion proteins were eluted from the columns, but no hUbc9 protein was found in the eluate (lane 4 and 8 of Fig. 3-14). The results showed that hUbc9 did not bind to the N- or C-terminal fragments of hRad18 in pull-down experiment, although the interactions of hUbc9 with hRad18C and hRad18N were found in yeast two-hybrid assays. The failure to show their interactions *in vitro* may be due to the weak interactions between them. A trace hUbc9 protein may be bound to the fusion proteins. A more sensitive method to detect their interactions, for example, using hUbc9 antibody to detect the bound hUbc9 protein, will be useful.

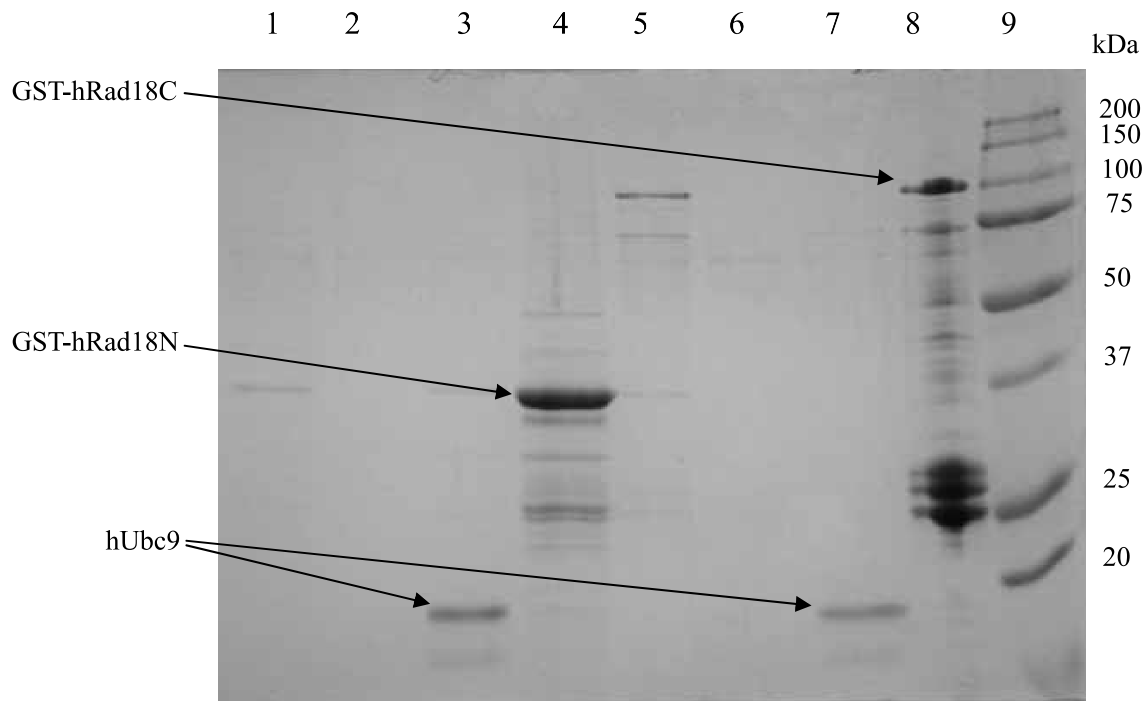

Fig. 3-14 SDS-PAGE analyses of pull-down experiments by GST-hRad18N (lane1-4) and GST-hRad18C (lane5-8) to bind hUbc9. Lane 1: flow-through of GST-hRad18N loaded onto MicroSpin GST column. Lane 2: flow-through of PBS wash. Lane 3: flow-through of loading hUbc9. Lane 4: Eluate. Lane 5: flow-through of GST-hRad18C loaded onto MicroSpin GST column. Lane 6: flow-through of PBS wash. Lane 7: flow-through of loading hUbc9. Lane 8: Eluate. Lane 9: protein molecular weight markers. The samples were run on a 12% polyacrylamide gel and the gel was then stained with Coomassie blue.

CHAPTER FOUR

DISCUSSION

4.1 Yeast two-hybrid assay is useful to study interactions between proteins or peptides

The yeast two-hybrid system has proven invaluable for identifying interactions between proteins since its introduction (Chien *et al.*, 1991; Fields and Song, 1989). In this study, we found that both the N- and C-terminal fragments of hRad18 can interact with hUbc9, the C-terminus also interacted with HHR6B by yeast two-hybrid assays. These interactions were not detected by *in vitro* binding experiments. One explanation is that the *in vitro* assay is not sensitive enough to detect the interactions and the quality of purified proteins may affect the sensitivity of this method. Another reason may be that the associations were transient or weak. In E2-E3 complex, the interaction between these two parts is likely unable to be detected by conventional studies such as coimmunoprecipitation (Brzovic *et al.*, 2003; Wooff *et al.*, 2004). Weak interaction in the complex may be necessary for quick dissociation of E2 from the complex and transfer of Ub/SUMO to substrate. Yeast two-hybrid analysis provides a way to investigate weak and probably transient interactions which are not feasible for conventional assays.

4.2 Expression of high-purity proteins to improve *in vitro* protein binding assays' sensitivity

In the study, a large amount of HHR6B and hUbc9 fusion proteins were yielded in *E. coli*. GST-hRad18N was expressed in an inclusion body form and was subsequently recovered by denaturation and refolding. The induced expression level of GST-hRad18C is low. The purified GST-hRad18C contained a lot of impurities even after stringent washes. GST-hRad18C was supposed to be able to pull down free HHR6B and Ubc9 proteins by *in vitro* experiments, whereas GST-hRad18N was supposed to pull down hUbc9. In my pull-down experiments, both of them failed. The reason may be that the affinity of the hRad18 fragments for HHR6B and hUbc9 are too low to bind them. Another possibility is that GST-hRad18N recovered from inclusion body folded incorrectly thus its binding to hUbc9 was changed. There are lots of impurities in purified GST-hRad18C, which may affect its binding to HHR6B and hUbc9. To get a good purity of human Rad18 protein and protein fragments is a way to improve the sensitivity of *in vitro* binding experiments.

E. coli is a successful and the preferred host for recombinant protein expression, but it is frequently not capable of expressing soluble heterologous proteins. The Southeast Collaboratory for Structural Genomics (SECSG) reports that of the 6386 proteins they have expressed in *E. coli* only 22.7% (1452) have been soluble (SECSG web page). Rad18 is proved to be an “unfriendly” protein to be expressed in soluble form or in large amount. I tried different ways to express yeast Rad18 in *E. coli*, yeast and baculovirus-infected insect cells, but with no success (data not shown). Recently, Ub (Baker, 1996; Hondred *et al.*, 1999) and SUMO (Butt *et al.*, 2005; Malakhov *et al.*, 2004;

Zuo *et al.*, 2005) have been used as a fusion partner to express recombinant proteins. Ub and SUMO fusion dramatically improve the expression and solubility of heterologous proteins in both prokaryotes and eukaryotes. Ub and SUMO are removed from fusion proteins by de-ubiquitinating enzymes (DUBs) and SUMO protease respectively. While DUBs are unstable and are difficult to produce, SUMO protease is easy to produce in large quantities and is able to cleave SUMO fusion proteins robustly and with impeccable specificity. It is promising to produce full length and truncated human Rad18 by using SUMO fusion system. The active hRad18 protein and various fragments are useful to demonstrate the interactions of them with HHR6B and hUbc9.

4.3 Rad6 and human Ubc9 interact with Rad18 through different domains

. Structural analysis of the E2-E3 complex reveals that RING finger domains of E3s mediates physical interaction with E2s (Wooff *et al.*, 2004; Zheng *et al.*, 2000). Studies of yeast Rad18 and Rad6 interaction indicate that *S. cerevisiae* Rad18 forms a complex with Rad6 through a 40 aa region, named R6BD (Bailly *et al.*, 1997b). Yeast two-hybrid assay showed this 40 aa domain of Rad18 is sufficient to interact with Rad6. Human Rad18 has a homologous domain of yeast R6BD from aa 340 to 395. In yeast two-hybrid assays, R6BD deletion completely abolished the interaction between human Rad18 and HHR6B. R6BD is responsible for the interaction of Rad18 and Rad6 in both yeast and human. Recently, Miyase *et al.* reported that two Rad18 molecules interact through the Zinc

finger domain, and one Rad18 molecule is ubiquitinated by the other Rad18 molecule in a Rad6-dependent manner (Miyase *et al.*, 2005). When Cys28 was mutated to Phe, Rad18 protein could still be ubiquitinated, suggesting that the RING finger domain is not involved in interaction with Rad6. The RING finger domain consists of a short motif rich in cysteine and histidine residues, which coordinate two zinc ions (Borden, 2000). The zinc ions and their ligands are catalytically inert; moreover, it is the spacing of the zinc ligands, rather than any primary sequence, that is conserved in the RING finger domain. These features suggest that RING finger domains function as molecular scaffolds that bring proteins together. Many RING finger domains have been shown to directly bind E2s (Albert et al., 2002; Brzovic et al., 2003; Wooff et al., 2004; Zheng et al., 2002; Zheng et al., 2000). In the interaction of Rad18-Rad6, a typical E2-E3 complex, the RING finger domain does not function as a binding domain. Its actual role needs to be further defined.

Human Ubc9 is an E2, specific for SUMO conjugation. Analysis of Ubc9 structure revealed important differences when compared with other Ub-conjugating enzymes. Within the amino-terminal helix both structural and sequence alignments do not match to Ub-conjugating enzymes due to one mismatched amino acid, which confers a different recognition surface on Ubc9 (Liu *et al.*, 1999a; Liu *et al.*, 1999b). It is not surprising that hUbc9 interacts with hRad18 in a different way from the interaction between hRad18 and HHR6B. From the yeast two-hybrid assays, there are two domains in hRad18 interacting with hUbc9. The first interacting domain is located at the

N-terminus of hRad18. Because the N-terminal fragment of hRad18 (hRad18N) is only 93 aa long and contains a RING finger domain, RING finger domain is possibly one the two domains of hRad18 interacting with hUbc9. The second domain is at the C-terminus, but the precise position is not clear. R6BD is at least not required for these two proteins' interactions because the C-terminal fragment without R6BD (hRad18CΔ6BD) can still interact with hUbc9. However, the exact domains of hRad18 interacting with hUbc9 need to be further elucidated.

Although hRad18C interacts with hUbc9, a key Cys28 mutation in the RING finger domain of hRad18 (hRad18C28F) abolished its interaction with hUbc9. Combined with the results that this Cys mutation eliminated the interaction between Rad18 and Rad6 (both yeast and human), in which the RING finger domain is not required for the interaction, it is reasonable to infer that Cys28 mutation may lead to instability and rapid degradation of Rad18 *in vivo*. This hypothesis was supported by the observation in an *in vitro* ubiquitination system (Miyase *et al.*, 2005). This system contains E1, E2, Rad18 or Rad18 mutants, and 26S proteasomal extract. After 24-hour incubation, Rad18C28F was not detected by a Rad18 antibody. By adding a proteasome inhibitor, non-, mono-, and poly-ubiquitinated Rad18 was detected. These results suggested that a RING finger mutant of Rad18 is an unstable protein and subject to degradation by proteasome.

4.4 Why does human Rad18 interact with Ubc9?

In this study, the interaction between hRad18 and hUbc9 was established. Ubc9

is the only E2 for sumoylation. Steps involved in SUMO modification parallel those involved in ubiquitination. As a RING finger E3, hRad18 forms a complex with Rad6 and they together mono-ubiquitinate PCNA. What is the biological function of the interaction between hRad18 and hUbc9? One hypothesis is that hRad18 serves as an adaptor protein to load hUbc9 onto the DNA replication fork where hUbc9 conjugates SUMO to PCNA. If hRad18 brings hUbc9 and PCNA together, it functions like a sumoylation E3. The difference between hRad18 and sumoylation E3s is that sumoylation E3s increase the substrate specificity by recognizing substrate directly, while hRad18 promotes PCNA sumoylation by recruiting hUbc9 to DNA strand through its ssDNA binding activity. Sumoylation has its own E3s, which are Siz1 and Siz2 in yeast (Johnson and Gupta, 2001; Takahashi *et al.*, 2001). PCNA can be SUMO modified in the absence of Rad18 (Papouli et al., 2005). It seems that Rad18 does not play a role in the sumoylation of PCNA. Another hypothesis is that when DNA damage occurs, Rad18 binds to ssDNA and interacts with Rad6 and Ubc9 simultaneously through different domains, thus preventing PCNA sumoylation in order to make PCNA available for ubiquitination. How hRad18 regulates its interaction with hRad6 or hUbc9 and affects the modification status of PCNA is the issue that needs to be further addressed.

4.5 Conclusions

The interactions between hRad18 full sequence protein, hRad18 truncated fragments, or hRad18 mutants and HHR6B/hUbc9 analyzed by yeast two-hybrid assays

were summarized in **Fig. 3-4**. From this study, several conclusions can be drawn:

(1) A small fragment of yeast and human Rad18 protein is required for interaction with Rad6. Yeast two-hybrid assays showed that the fragment consisting of residues 371 to 410 of yeast Rad18 is sufficient to interact with Rad6, and that deletion of the homologous fragment in human Rad18 (from residues 340 to 395) abolished the interaction between hRad18 and HHR6B.

(2) Interaction between human Ubc9 and Rad18 was found in yeast two-hybrid assay. There are two domains in human Rad18 interacting with Ubc9. One is located at the N-terminus, the other is located at the C-terminus of human Rad18. The interactions between Ubc9 and the N- and C-terminal fragments of Rad18 need to be confirmed *in vitro*.

(3) A key Cysteine mutation in RING finger domain of yeast and human Rad18 abolished all interactions of Rad18 with Rad6 and Ubc9. Combined with Miyase's result, the RING finger mutant of Rad18 is believed to produce an unstable protein, subject to degradation.

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